

TO7290-32CT6860

Polymerases for Analyzing or Typing Polymorphic Nucleic Acid Fragments and Uses Thereof

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application No. 60/037,393, filed February 7, 1997, and to the U.S. Provisional Application of Deb K. Chatterjee, Joseph Solus and Shuwei Yang, entitled "Polymerases for Analyzing or Typing Polymorphic Nucleic Acid Fragments and Uses Thereof," filed January 6, 1998, the disclosures of which are fully incorporated herein by reference.

10

FIELD OF THE INVENTION

The present invention is in the field of molecular and cellular biology. The invention relates to compositions and methods for use in analyzing and typing polymorphic regions of DNA. More particularly, the invention is directed to compositions of polymerases (preferably DNA polymerases and most preferably thermostable DNA polymerases), and methods using these compositions, whereby polymorphic, minisatellite, microsatellite or STR DNA fragments may be amplified and analyzed. The compositions and methods of the present invention are useful in a variety of techniques employing DNA amplification and polymorphism analysis, including medical genetic, forensic, and plant breeding applications.

20

The present invention also relates to polymerases having reduced, substantially reduced or eliminated ability to add one or more non-templated nucleotides to the 3' terminus of a synthesized nucleic acid molecule. Preferably, the polymerases of the invention are thermostable or mesophilic polymerases. Specifically, the polymerases of the present invention (e.g., DNA or RNA polymerases) have been mutated or modified to reduce, substantially reduce or eliminate such activity (compared to the unmodified, unmutated, or wild type polymerase), thereby providing a polymerase which synthesizes nucleic acid molecules having little or no non-templated 3' terminal nucleotides. Such

25

polymerases thus have enhanced or greater ability to produce a double stranded nucleic acid molecule having blunt ended termini which may facilitate cloning of such molecules. The present invention also relates to cloning and expression of the polymerases of the invention, to nucleic acid molecules containing the cloned genes, and to host cells which express said genes. The polymerases of the present invention may be used in DNA sequencing, amplification, nucleic acid synthesis, and polymorphism analysis.

The invention also relates to polymerases of the invention which have one or more additional mutations or modifications. Such mutations or modifications include those which (1) substantially reduce 3'→5' exonuclease activity; and/or (2) substantially reduce 5'→3' exonuclease activity. The polymerases of the invention can have one or more of these properties. These polymerases may also be used in nucleic acid analysis including but not limited to DNA sequencing, amplification, nucleic acid synthesis, and polymorphism analysis.

BACKGROUND OF THE INVENTION

DNA Structure

The genetic framework (*i.e.*, the genome) of an organism is encoded in the double-stranded sequence of nucleotide bases in the deoxyribonucleic acid (DNA) which is contained in the somatic and germ cells of the organism. The genetic content of a particular segment of DNA, or gene, is only manifested upon production of the protein which the gene ultimately encodes. There are additional sequences in the genome that do not encode a protein (*i.e.*, "noncoding" regions) which may serve a structural, regulatory, or unknown function. Thus, the genome of an organism or cell is the complete collection of protein-encoding genes together with intervening noncoding DNA sequences. Importantly, each somatic cell of a multicellular organism contains the full complement of genomic DNA of the organism, except in cases of focal infections or cancers, where one or more

xenogeneic DNA sequences may be inserted into the genomic DNA of specific cells and not into other, non-infected, cells in the organism.

Minisatellite and Microsatellite DNA

Interspersed throughout the genomic DNA of most eukaryotic organisms are short stretches of polymorphic repetitive nucleotide sequences known as "minisatellite DNA" sequences or fragments (Jeffreys, A.J., *et al.*, *Nature* 314:67-73 (1985)). These repeating sequences often appear in tandem and in variable numbers within the genome, and they are thus sometimes referred to as "short tandem repeats" ("STRs") or "variable numbers of tandem repeats" ("VNTRs") (see U.S. Patent No. 5,075,217; Nakamura *et al.*, *Science* 235:1616-1622 (1987)). Typically, however, minisatellite repeat units are about 9 to 60 bases in length (Nakamura *et al.*, *Science* 235:1616-1622 (1987); Weber and May, *Am. J. Hum. Genet.* 44:388-396 (1989)) which are repeated in tandem about 20-50 times (Watson, J.D., *et al.*, eds., *Recombinant DNA*, 2nd ed., New York: Scientific American Books, p. 146 (1992)). Other short, simple sequences which are analogous to minisatellite DNAs, termed "microsatellite DNAs" (Litt, M., and Luty, J.A., *Am. J. Hum. Genet.* 44:397-401 (1989); Weber and May, *Am. J. Hum. Genet.* 44:388-396 (1989)), are usually about 1-6 bases in repeat unit length and thus give rise to monomeric (Economou, E.T., *et al.*, *Proc. Natl. Acad. Sci. USA* 87:2951-2954 (1990)), dimeric, trimeric, quatrameric, pentameric or hexameric repeat units (Litt, M., and Luty, J.A., *Am. J. Hum. Genet.* 44:397-401 (1989); Weber and May, *Am. J. Hum. Genet.* 44:388-396 (1989)). The most prevalent of these highly polymorphic microsatellite sequences in the human genome is the dinucleotide repeat (dC-dA)_n(dG-dT)_n (where n is the number of repetitions in a given stretch of nucleotides), which is present in a copy number of about 50,000-100,000 (Tautz, D., and Renz, M., *Nucl. Acids Res.* 12:4127-4138 (1984); Dib, C., *et al.*, *Nature* 360:152-154 (1996)), although the existence of a variety of analogous repeat sequences in the genomes of evolutionarily diverse eukaryotes

has been reported (Hamada, H., *et al.*, *Proc. Natl. Acad. Sci. USA* 79:6465-6469 (1982)).

The actual *in vivo* function of minisatellite and microsatellite sequences is unknown. However, because these tandemly repeated sequences are dispersed throughout the genome of most eukaryotes, exhibit size polymorphism, and are often heterozygous (Weber, J.L., *Genomics* 7:524-530 (1990)), they have been explored as potential genetic markers in assays attempting to distinguish closely related individuals, and in forensic and paternity testing (see, e.g., U.S. Patent No. 5,075,217; Jeffreys, A.J., *et al.*, *Nature* 332:278-281 (1988)). The finding that mutations often are observed in microsatellite DNA regions in cancer cells (Loeb, L.A., *Cancer Res.* 54:5059-5063 (1994)), potentially linking genomic instability to the carcinogenic process and providing useful genetic markers of cancer, lends additional significance to methods facilitating the rapid analysis and genotyping of polymorphisms in these genomic DNA regions.

Methods of Genotyping Minisatellite or STR DNA Sequences

To analyze minisatellite, microsatellite or STR DNA sequence polymorphisms, a variety of molecular biological techniques have been employed. These techniques include restriction fragment length polymorphism (RFLP) or "DNA fingerprinting" analysis (Wong, Z., *et al.*, *Nucl. Acids Res.* 14:4605-4616 (1986); Wong, Z., *et al.*, *Ann. Hum. Genet* 51:269-288 (1987); Jeffreys, A.J., *et al.*, *Nature* 332:278-281 (1988); U.S. Patent Nos. 5,175,082; 5,413,908; 5,459,039; and 5,556,955). Far more commonly employed for STR genotyping than RFLP and hybridization, however, are amplification-based methods, such as those relying on the polymerase chain reaction (PCR) method invented by Mullis and colleagues (see U.S. Patent Nos. 4,683,195; 4,683,202; and 4,800,159). These methods use "primer" sequences which are complementary to opposing regions flanking the polymorphic DNA sequence to be amplified from the sample of genomic DNA to be analyzed. These primers are added to the DNA target sample, along with excess deoxynucleotides and a DNA polymerase (e.g., *Taq*

TO2009-0007560

polymerase; see below), and the primers bind to their target via base-specific binding interactions (*i.e.*, adenine binds to thymine, cytosine to guanine). By repeatedly passing the reaction mixture through cycles of increasing and decreasing temperatures (to allow dissociation of the two DNA strands on the target sequence, synthesis of complementary copies of each strand by the polymerase, and re-annealing of the new complementary strands), the copy number of the minisatellite or STR sequence of DNA may be rapidly increased, and detected by size separation methods such as gel electrophoresis.

PCR and related amplification approaches have been used in attempts to develop methods for typing and analyzing STRs or minisatellite regions. For example, PCR has been employed to analyze polymorphisms in microsatellite sequences from different individuals, including $(dC-dA)n \cdot (dG-dT)n$ (Weber, J.L., and May, P.E., *Am. J. Hum. Genet.* 44:388-396 (1989); Weber, J. L., *Genomics* 7:524-530 (1990); U.S. Patent Nos. 5,075,217; 5,369,004; and 5,468,613). Similar methods have been applied to a variety of medical and forensic samples to perform DNA typing and to detect polymorphisms between individual samples (U.S. Patent Nos. 5,306,616; 5,364,759; 5,378,602; and 5,468,610).

In Vitro Use of DNA Polymerases

The above-described amplification-based techniques require the use of DNA polymerases, which catalyze the addition of deoxynucleoside triphosphate (dNTP) bases into the newly forming DNA strands. Together with other enzymes (*e.g.*, helicases, ligases and ATPases), the DNA polymerases ensure rapid and relatively faithful replication of DNA in preparation for proliferation *in vivo* in prokaryotes, eukaryotes and viruses.

DNA polymerases synthesize the formation of DNA molecules which are complementary to a DNA template. Upon hybridization of a primer to the single-stranded DNA template, polymerases synthesize DNA in the 5' to 3' direction, successively adding nucleotides to the 3'-hydroxyl group of the growing strand. Thus, in the presence of deoxyribonucleoside triphosphates (dNTPs) and a primer,

a new DNA molecule, complementary to the single stranded DNA template, can be synthesized.

In addition to an activity which adds dNTPs to DNA in the 5' to 3' direction (*i.e.*, "polymerase" activity), many DNA polymerases also possess activities which remove dNTPs in the 5' to 3' and/or the 3' to 5' direction (*i.e.*, "exonuclease" activity). This dual activity of certain DNA polymerases is, however, a drawback for some *in vitro* applications. For example, the *in vitro* synthesis of an intact copy of a DNA fragment by the polymerase activity, an elongation process which proceeds in a 5' to 3' direction along the template DNA strand, is jeopardized by the exonuclease activities which may simultaneously or subsequently degrade the newly formed DNA.

Limitations of PCR-based Genotyping of Minisatellite, Microsatellite and STR DNA Sequences

Application of PCR-based methods to analysis of minisatellite or STR DNA sequences has a number of significant limitations. It has been shown, for example, that use of *Tag* and other thermostable DNA polymerases commonly employed in PCR and related automated amplification methods causes the accumulation of amplification products containing non-templated 3' terminal nucleotides (Clark, J.M., *et al.*, *J. Molec. Biol.* 198:123-127 (1987); Clark, J.M., *Nucl. Acids Res.* 16:9677-9686 (1988); Hu, G., *DNA Cell Biol.* 12:763-770 (1993)). That is, some of the newly synthesized DNA strands produced in each round of amplification have had an extra nucleotide added to their 3' termini, such that the newly synthesized strands may be longer by one base.

Non-templated nucleotide addition is a slow process compared to template-directed synthesis (Clark, J.M., *Nucl. Acids Res.* 16:9677-9686 (1988)), and its extent is sequence-dependent (Hu, G., *DNA Cell Biol.* 12:763-770 (1993); Brownstein, M.J., *et al.*, *BioTechniques* 20:1004-1010 (1996)). Consequently, the PCR product is often heterogeneous in regard to extra nucleotide addition depending upon the primers and the reaction conditions used by the investigator

000001332-0529741

(Magnuson, V.L., *et al.*, *BioTechniques* 21:700-709 (1996)). Extra nucleotide addition, in combination with "stutter" due to slippage during PCR amplification (Levinson, G., and Gutman, G.A., *Molec. Biol. Evol.* 4:203-221 (1987); Schlotterer, C., and Tautz, D., *Nucl. Acids Res.* 20:211-215 (1992)), often results in complex DNA fragment patterns which are difficult to interpret, especially by automated methods. This can result in improper genotyping analysis, particularly if the percentage of non-templated nucleotide addition is between 30-70% of the PCR product (Smith, J.R., *et al.*, *Genome Res.* 5:312-317 (1995)).

Thus, a need currently exists for a rapid, automated method for identifying, analyzing and typing polymorphic DNA fragments, particularly minisatellite, microsatellite or STR DNA fragments, that will not result in the problematic results described above. The present invention provides such a method.

BRIEF SUMMARY OF THE INVENTION

The present invention satisfies these needs in the art by providing methods useful in the identification, analysis or typing of polymorphic DNA fragments, particularly minisatellite, microsatellite or STR DNA fragments, in samples of DNA from a cell, particularly a eukaryotic cell. Specifically, the invention provides a method of producing a population of amplified DNA molecules, for use in analyzing or typing a DNA molecule in a DNA sample isolated from a cell, preferably a eukaryotic cell. The method of the present invention comprises contacting a DNA sample with a DNA polymerase (preferably a thermostable DNA polymerases) reduced, substantially reduced or eliminated in the ability to add one or more non-templated nucleotides to the 3' terminus of a DNA molecule, amplifying a polymorphic DNA fragment, preferably a minisatellite, microsatellite or STR DNA fragment, within the DNA sample and analyzing the amplified polymorphic DNA fragment. In the method of the invention, the analysis step may comprise, for example, sizing or sequencing the amplified DNA molecule and optionally comparing the size and/or sequence of the amplified DNA molecule to

010701020304050607080900

a different DNA sample which has been amplified according to the invention. In preferred embodiments of the present invention, the thermostable DNA polymerase is a *Thermotoga* DNA polymerase, preferably a *Thermotoga* DNA polymerase substantially reduced in 3'-5' exonuclease activity, more preferably a 5 *Tne* polymerase, a *Tma* polymerase, or a mutant or derivative thereof, and most preferably a mutant of *Tne* polymerase selected from the group consisting of *Tne* N'Δ219, D323A; *Tne* N'Δ283, D323A; *Tne* N'Δ284, D323A; *Tne* N'Δ193, D323A; *Tne* D137A, D323A; *Tne* D8A, D323A; *Tne* G195D, D323A; *Tne* G37D, D323A; *Tne* N'Δ283; *Tne* D137A, D323A, R722K; *Tne* D137A, D323A, R722Y; 10 *Tne* D137A, D323A, R722L; *Tne* D137A, D323A, R722H; *Tne* D137A, D323A, R722Q; *Tne* D137A, D323A, F730Y; *Tne* D137A, D323A, K726R; *Tne* D137A, D323A, K726H; *Tne* D137A, D323A, R722K, F730Y; *Tne* D137A, D323A, R722K, F730Y; *Tne* D137A, D323A, R722K, F730Y; *Tne* D137A, D323A, R722H, F730Y; *Tne* D137A, D323A, R722H, K726R; *Tne* D137A, D323A, R722H, K726H; *Tne* D137A, D323A, R722H, K726R; *Tne* D137A, D323A, R722Q, F730Y; *Tne* D137A, D323A, R722Q, K726R; *Tne* D137A, D323A, R722Q, K726H; *Tne* D137A, D323A, R722N, F730Y; *Tne* D137A, D323A, R722N, K726R; *Tne* D137A, D323A, R722N, K726H; *Tne* D137A, D323A, F730S; *Tne* N'Δ283, D323A, R722K/H/Q/N/Y/L; *Tne* N'Δ219, D323A, R722K; *Tne* N'Δ219, D323A, F730Y; 15 *Tne* N'Δ219, D323A, K726R; *Tne* N'Δ219, D323A, K726H; *Tne* D137A, D323A, F730S, R722K/Y/Q/N/H/L, K726R/H; *Tne* D137A, D323A, F730T, R722K/Y/Q/N/H/L, K726R/H; *Tne* D137A, D323A, F730T; *Tne* F730S; *Tne* F730A; *Tne* K726R; *Tne* K726H; and *Tne* D137A, D323A, R722N. The present invention is particularly directed to the above methods wherein the eukaryotic cell 20 is a plant cell or an animal cell, preferably a mammalian cell, more preferably a normal, diseased, cancerous, fetal or embryonic mammalian cell, and most preferably a human cell. The invention is also directed to the above methods, further comprising isolating the polymorphic, minisatellite, microsatellite or STR DNA fragment and inserting it into a vector, preferably an expression vector. By 25

50098132 2022-05-27

the present methods, the polymorphic or microsatellite DNA fragment may be amplified prior to being inserted into the vector.

The present invention also provides a method of determining the relationship between a first individual and a second individual, comprising contacting a DNA sample from the first and second individuals with a DNA polymerase (e.g. a thermostable DNA polymerase) reduced, substantially reduced or eliminated in the ability to add one or more non-templated nucleotides to the 3' terminus of a DNA molecule, amplifying one or more DNA molecules in the DNA sample to generate a collection of amplified polymorphic DNA fragments, separating the amplified DNA fragments by length, and comparing the pattern of amplified DNA fragments from the first individual to that of the second individual. This method also allows the identification of one or more unique polymorphic DNA fragments, particularly a minisatellite, microsatellite or STR DNA fragment, that is specifically present in only one of the two individuals. This method may further comprise determining the sequence of the unique polymorphic, minisatellite, microsatellite or STR DNA fragment. In this embodiment of the present invention, the thermostable DNA polymerase may be a *Thermotoga* DNA polymerase, preferably a *Thermotoga* DNA polymerase substantially reduced in 3'-5' exonuclease activity, more preferably a *Tne* polymerase, a *Tma* polymerase, or a mutant or derivative thereof, and most preferably a mutant of *Tne* polymerase selected from the group consisting of *Tne* N'Δ219, D323A; *Tne* N'Δ283, D323A; *Tne* N'Δ284, D323A; *Tne* N'Δ193, D323A; *Tne* D137A, D323A; *Tne* D8A, D323A; *Tne* G195D, D323A; *Tne* G37D, D323A; *Tne* N'Δ283; *Tne* D137A, D323A, R722K; *Tne* D137A, D323A, R722Y; *Tne* D137A, D323A, R722L; *Tne* D137A, D323A, R722H; *Tne* D137A, D323A, R722Q; *Tne* D137A, D323A, F730Y; *Tne* D137A, D323A, K726R; *Tne* D137A, D323A, K726H; *Tne* D137A, D323A, R722K, F730Y; *Tne* D137A, D323A, R722K, K726R; *Tne* D137A, D323A, R722K, K726H; *Tne* D137A, D323A, R722H, F730Y; *Tne* D137A, D323A, R722H, K726R; *Tne* D137A, D323A, R722Q, F730Y; *Tne* D137A, D323A, R722Q, K726R; *Tne* D137A,

TO BE ENTERED
BY
TODAY'S
PUBLICATION
DATE

D323A, R722Q, K726H; *The* D137A, D323A, R722N, F730Y; *The* D137A, D323A, R722N, K726R; *The* D137A, D323A, R722N, K726H; *The* D137A, D323A, F730S; *The* N'Δ283, D323A, R722K/H/Q/N/Y/L; *The* N'Δ219, D323A, R722K; *The* N'Δ219, D323A, F730Y; *The* N'Δ219, D323A, K726R; *The* N'Δ219, 5 D323A, K726H; *The* D137A, D323A, F730S, R722K/Y/Q/N/H/L, K726R/H; *The* D137A, D323A, F730T, R722K/Y/Q/N/H/L, K726R/H; *The* D137A, D323A, F730T; *The* F730S; *The* F730A; *The* K726R; *The* K726H; and *The* D137A, D323A, R722N. The present invention is particularly directed to the above methods wherein the first or second individual is an animal or a plant, and most preferably wherein the first or second individual is a human.

The present invention also provides isolated nucleic acid molecules encoding mutant *The* DNA polymerase proteins, wherein the mutant *The* DNA polymerase proteins have an amino acid sequence as set forth in any one of SEQ ID NOs: 4-10. The invention also provides mutant *The* DNA polymerase proteins having an amino acid sequence as set forth in any one of SEQ ID NOs:4-10, most preferably a mutant *The* polymerase protein selected from the group consisting of *The* N'Δ283, D323A (SEQ ID NO:4); *The* N'Δ193, D323A (SEQ ID NO:5); *The* D137A, D323A (SEQ ID NO:6); *The* D8A, D323A (SEQ ID NO:7); *The* G195D, D323A (SEQ ID NO:8); *The* G37D, D323A (SEQ ID NO:9); and *The* N'Δ283 (SEQ ID NO:10). The invention also relates to nucleic acid molecules and the proteins encoded by such nucleic acid molecules for mutant *The* polymerases selected from the group consisting of *The* n'Δ283; *The* D137A, D323A, R722K; *The* D137A, D323A, R722Y; *The* D137A, D323A, R722L; *The* D137A, D323A, R722H; *The* D137A, D323A, R722Q; *The* D137A, D323A, F730Y; *The* D137A, D323A, K726R; *The* D137A, D323A, K726H; *The* D137A, D323A, R722K, F730Y; *The* D137A, D323A, R722K, K726R; *The* D137A, D323A, R722K, K726H; *The* D137A, D323A, R722H, F730Y; *The* D137A, D323A, R722H, K726R; *The* D137A, D323A, R722H, K726H; *The* D137A, D323A, R722Q, F730Y; *The* D137A, D323A, R722Q, K726R; *The* D137A, D323A, R722Q, K726H; *The* D137A, D323A, R722N, F730Y; *The* D137A, 10 15 20 25 30

D323A, R722N, K726R; *The* D137A, D323A, R722N, K726H; *The* D137A, D323A, F730S; *The* N^Δ283, D323A, R722K/Y/Q/N/Y/L; *The* N^Δ219, D323A, R722K; *The* N^Δ219, D323A, F730Y; *The* N^Δ219, D323A, K726R; *The* N^Δ219, D323A, K726H; *The* D137A, D323A, F730S, R722K/Y/Q/N/H/L, K726R/H; *The* D137A, D323A, F730T, R722K/Y/Q/N/H/L, K726R/H; *The* D137A, D323A, F730T; *The* F730S; *The* F730A; *The* K726R; *The* K726H; and *The* D137A, D323A, R722N. These mutations may be made to sequence ID NO:2 to produce the mutant polymerases having the indicated amino acid mutations (where, for example, "D137A" indicates that the Asp (D) residue at position 137 in SEQ ID NO:2 has been mutated to an Ala (A) residue, and, for example, "R722K/Y/Q/N/H/L" indicates that the Arg (R) residue at position 722 in SEQ ID NO:2 has been mutated to a Lys (K), Tyr (Y), Gln (Q), Asn (N), His (H) or Leu (L) residue).

The present invention also provides kits for the identification, analysis or typing of a polymorphic DNA fragment, particularly a minisatellite, microsatellite or STR DNA fragment, comprising a first container containing one or more DNA polymerases reduced, substantially reduced or eliminated in the ability to add non-templated 3' terminal nucleotides. Kits according to the invention may contain additional containers selected from the group consisting of a container containing one or more DNA primer molecules, a container containing one or more deoxynucleoside triphosphates needed to synthesize a DNA molecule complementary to the DNA template, and a container containing a buffer suitable for identifying, analyzing or typing a polymorphic DNA fragment by the methods of the invention. Any number of these components of the kit may be combined in a single or multiple containers to provide the kit of the invention. According to the invention, the DNA polymerase of the kit is preferably a *Thermotoga* DNA polymerase, more preferably a *Thermotoga* DNA polymerase substantially reduced in 3'-5' exonuclease activity, still more preferably a *The* polymerase, a *Tma* polymerase, or a mutant or derivative thereof, and most preferably a mutant of *The* polymerase selected from the group consisting of *The* N^Δ283; *The* D137A,

TO2290-32576860

5

10

15

20

25

30

5 D323A, R722K; *Tne* D137A, D323A, R722Y; *Tne* D137A, D323A, R722L;
10 *Tne* D137A, D323A, R722H; *Tne* D137A, D323A, R722Q; *Tne* D137A, D323A,
F730Y; *Tne* D137A, D323A, K726R; *Tne* D137A, D323A, K726H; *Tne* D137A,
D323A, R722K, F730Y; *Tne* D137A, D323A, R722K, K726R; *Tne* D137A,
D323A, R722K, K726H; *Tne* D137A, D323A, R722H, F730Y; *Tne* D137A,
D323A, R722H, K726R; *Tne* D137A, D323A, R722H, K726H; *Tne* D137A,
D323A, R722Q, F730Y; *Tne* D137A, D323A, R722Q, K726R; *Tne* D137A,
D323A, R722Q, K726H; *Tne* D137A, D323A, R722N, F730Y; *Tne* D137A,
D323A, R722N, K726R; *Tne* D137A, D323A, R722N, K726H; *Tne* D137A,
D323A, F730S; *Tne* N'Δ283, D323A, R722K/H/Q/N/Y/L; *Tne* N'Δ219, D323A,
R722K; *Tne* N'Δ219, D323A, F730Y; *Tne* N'Δ219, D323A, K726R; *Tne* N'Δ219,
D323A, K726H; *Tne* D137A, D323A, F730S, R722K/Y/Q/N/H/L, K726R/H; *Tne*
15 D137A, D323A, F730T, R722K/Y/Q/N/H/L, K726R/H; *Tne* D137A, D323A,
F730T; *Tne* F730S; *Tne* F730A; *Tne* K726R; *Tne* K726H, and *Tne* D137A,
D323A, R722N.

20 The present invention also relates generally to mutated or modified polymerases (DNA or RNA polymerases) which have reduced, substantially reduced or eliminated ability to add one or more non-templated nucleotides to the 3' terminus of a synthesized nucleic acid molecule (compared to the corresponding wildtype, unmutated or unmodified polymerase). Preferably, such mutant or modified polymerases have substantially reduced ability to add one or more non-templated nucleotides to the 3' terminus of a synthesized nucleic acid molecule.
25 Such polymerases of the invention may be thermostable or mesophilic polymerases. Thus, the present invention relates to such mutated or modified polymerases and to kits containing such polymerases. The invention also relates to the use of such mutant or modified polymerases in a number of procedures including DNA sequencing, amplification reactions, nucleic acid synthesis, and polymorphism analysis.

25 Mutant or modified polymerases of particular interest in the invention include *Taq* DNA polymerase, *Tne* DNA polymerase, *Tma* DNA polymerase, *Pfu*

162298-20076360

DNA polymerase, *Tfl* DNA polymerase, *Tth* DNA polymerase, *Tbr* DNA polymerase, *Pwo* DNA polymerase, *Bst* DNA polymerase, *Bca* DNA polymerase, VENT™ DNA polymerase, DEEP VENT™ DNA polymerase, T7 DNA polymerase, T5 DNA polymerase, DNA polymerase III, Klenow fragment DNA polymerase, Stoffel fragment DNA polymerase, and mutants, fragments or derivatives thereof. RNA polymerases of interest include T7, SP6, and T3 RNA polymerases and mutants, variants and derivatives thereof.

The present invention relates in particular to mutant PolI type DNA polymerases (preferably thermostable DNA polymerases) wherein one or more mutations have been made in the O-helix which reduces, substantially reduces or eliminates the ability of the enzyme to add one or more non-templated nucleotides to the 3' terminus of a synthesized nucleic acid molecule. The O-helix is defined as RXXXKXXXFXXXYX (SEQ ID NO:11), wherein X may be any amino acid. The preferred sites for mutation or modification to produce the polymerases of the invention are the R and/or F and/or K and/or Y positions in the O-helix, although other changes (or combinations thereof) within the O-helix may be made to make the desired polymerase. In this preferred aspect of the invention, R and/or F and/or K and/or Y may be replaced with any other amino acid including Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val.

In accordance with the invention, other functional changes (or combinations thereof) may be made to the polymerases having reduced ability to add non-templated nucleotides to the 3' terminus of a synthesized nucleic acid molecule. For example, the polymerase may also be modified to reduce, substantially reduce or eliminate 5' exonuclease activity, and/or 3' exonuclease activity. Thus, the invention relates to mutant or modified DNA polymerases having reduced ability to add non-templated nucleotides which are modified in at least one way selected from the group consisting of

(a) to reduce or eliminate the 3'-5' exonuclease activity of the polymerase;

100-220-322-580

5

10

15

20

25

30

(b) to reduce or eliminate the 5'-3' exonuclease activity of the polymerase;

Any one or a number of these mutations or modifications (or combinations thereof) may be made to provide the polymerases of the invention. Preferred polymerases of the invention, in addition to having reduced ability to add non-templated 3' nucleotides, also have reduced, substantially reduced or eliminated 3' exonuclease activity.

The present invention is also directed to nucleic acid molecules (preferably vectors) containing a gene encoding the mutant or modified polymerases of the present invention and to host cells containing such molecules. Any number of hosts may be used to express the gene of interest, including prokaryotic and eukaryotic cells. Preferably, prokaryotic cells are used to express the polymerases of the invention. The preferred prokaryotic host according to the present invention is *E. coli*.

The invention also relates to a method of producing the polymerases of the invention, said method comprising:

- (a) culturing the host cell comprising a gene encoding a polymerase of the invention;
- (b) expressing said gene; and
- (c) isolating said polymerase from said host cell.

The invention also relates to a method of synthesizing a nucleic acid molecule comprising:

- (a) mixing one or more nucleic acid templates (e.g. RNA or DNA) with one or more polymerases of the invention; and
- (b) incubating said mixture under conditions sufficient to synthesize nucleic acid molecules complementary to all or a portion of said templates. Such condition may include incubation with one or more deoxy- and/or dideoxyribonucleoside triphosphates. Such deoxy- and dideoxyribonucleoside triphosphates include dATP, dCTP, dGTP, dTTP, dITP, 7-deaza-dGTP, 7-deaza-dATP, dUTP, ddATP, ddCTP, ddGTP, ddITP, ddTTP, [α -S]dATP, [α -S]dTTP,

[α -S]dGTP, and [α -S]dCTP. The synthesized nucleic acid molecules may in accordance with the invention be cloned into one or more vectors.

The invention also relates to a method of sequencing a DNA molecule, comprising:

5 (a) hybridizing a primer to a first DNA molecule;

(b) contacting said molecule of step (a) with deoxyribonucleoside triphosphates, one or more DNA polymerases of the invention, and one or more terminator nucleotides;

10 (c) incubating the mixture of step (b) under conditions sufficient to synthesize a random population of DNA molecules complementary to said first DNA molecule, wherein said synthesized DNA molecules are shorter in length than said first DNA molecule and wherein said synthesized DNA molecules comprise a terminator nucleotide at their 3' termini; and

15 (d) separating said synthesized DNA molecules by size so that at least a part of the nucleotide sequence of said first DNA molecule can be determined. Such terminator nucleotides include but are not limited to dideoxyribonucleoside triphosphates such as ddTTP, ddATP, ddGTP, ddITP or ddCTP.

The invention also relates to a method for amplifying a double stranded DNA molecule, comprising:

20 (a) providing a first and second primer, wherein said first primer is complementary to a sequence at or near the 3'-termini of the first strand of said DNA molecule and said second primer is complementary to a sequence at or near the 3'-termini of the second strand of said DNA molecule;

25 (b) hybridizing said first primer to said first strand and said second primer to said second strand in the presence of one or more polymerases of the invention, under conditions such that a third DNA molecule complementary to said first strand and a fourth DNA molecule complementary to said second strand are synthesized;

30 (c) denaturing said first and third strands, and said second and fourth strands; and

102390120016860

(d) repeating steps (a) to (c) one or more times. The amplified double-stranded nucleic acid molecules produced by the method of the invention may be cloned into one or more vectors. Thus, the invention relates also to a method of cloning an amplified DNA molecule comprising:

5 (a) amplifying one or more DNA molecules with one or more polymerases of the invention; and

(b) ligating said amplified DNA molecules in one or more vectors.

The invention further relates to a method of cloning a nucleic acid molecule comprising:

10 (a) mixing a nucleic acid template (or one or more templates) with one or more polymerases of the invention;

(b) incubating said mixture under conditions sufficient to synthesize a nucleic acid molecule complementary to all or a portion of said template, thereby producing a double-stranded nucleic acid molecule (preferably a double-stranded DNA molecule); and

(c) ligating said double-stranded nucleic acid molecule into one or more vectors.

Preferably, the vectors used for ligating the amplified or synthesized double-stranded nucleic acid molecules have blunt ended termini and may be prepared by digesting a vector with any one or a number of restriction enzymes known in the art which provide blunt end cleavage. Such restriction enzymes include *ScalI*, *SmaI*, *HpaI*, *HincII*, *HaeIII*, *AluI*, and the like.

The invention also relates to kits for sequencing, amplifying, synthesizing or cloning of nucleic acid molecules comprising one or more polymerases of the invention and one or more other components (or combinations thereof) selected from the group consisting of

25 (a) one or more dideoxyribonucleoside triphosphates;

(b) one or more deoxyribonucleoside triphosphates;

(c) one or more primers;

(d) one or more suitable buffers; and

(e) one or more ligases.

Other preferred embodiments of the present invention will be apparent to one of ordinary skill in light of the following drawings and description of the invention, and of the claims.

5

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1 shows the restriction map of the approximate DNA fragment which contains the *Tne* DNA polymerase gene in pSport 1 and pUC19. This figure also shows the region containing the O-helix homologous sequences.

FIGURE 2A schematically depicts the construction of plasmids pUC-Tne (3'→5') and pUC-Tne FY.

FIGURE 2B schematically depicts the construction of plasmids pTrcTne35 and pTrcTne FY.

FIGURE 3 schematically depicts the construction of plasmid pTrcTne35 FY.

FIGURE 4 schematically depicts the construction of plasmids pTTQTne5FY and pTTQTne535FY.

FIGURE 5 depicts a plasmid containing the *Taq* DNA polymerase gene.

FIGURE 6 depicts an autoradiogram showing of the ability of polymerase mutants to add non-templated 3' nucleotides.

102290-285145865

FIGURE 7 is an autoradiogram of the product of PCR amplification of the upper and lower alleles of the CD4 locus, using primers corresponding to these alleles, demonstrating nontemplated nucleotide addition (n+1) by *Taq* DNA polymerase but not by *Tne* DNA polymerase.

5 FIGURE 8 is an autoradiogram of the product of PCR amplification of the upper and lower alleles of the D20S27 locus, using primers corresponding to these alleles, demonstrating nontemplated nucleotide addition (n+1) by *Taq* DNA polymerase but not by *Tne* DNA polymerase.

10 FIGURE 9 is a composite of electropherogram gel scans of PCR amplifications at the D15S153 (Figures 9A and 9B) and D15S127 loci (Figures 9C and 9D), demonstrating nontemplated nucleotide addition (n+1) by *Taq* DNA polymerase (Figures 9A and 9C) but not by *Tne* DNA polymerase (Figures 9B and 9D).

15 FIGURE 10A and B are composites of a electropherogram gel scan of PCR amplifications at D16S405 and D16S401 loci.

FIGURE 11 is a composite of a electropherogram gel scan of PCR amplifications at D16S401 locus.

FIGURE 12A and B are composites of a electropherogram gel scan of PCR amplifications at D15S127 and D15S153 loci.

20 FIGURE 13 is a composite of a electropherogram gel scan of PCR amplifications at D16S401 locus.

TQZ290-22516860

DETAILED DESCRIPTION OF THE INVENTION

Definitions

In the description that follows, a number of terms used in recombinant DNA technology are extensively utilized. In order to provide a clearer and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

10 Polymorphic. As is understood by one of ordinary skill in the art, a nucleic acid molecule is said to be "polymorphic" if it may exist in more than one form. For example, a nucleic acid molecule is said to be polymorphic if it may have more than one specific nucleotide sequence (such as degenerate nucleic acid molecules or genes that may each encode the same protein). More commonly, a nucleic acid molecule is said to be polymorphic if it displays size differences (*i.e.*, differences in length), particularly when comparisons of nucleic acid molecules from different individuals are made. Of course, other definitions of the term "polymorphic" will be apparent to one of ordinary skill and are also encompassed within this definition.

15 Cloning vector. A plasmid, cosmid or phage DNA or other DNA molecule which is able to replicate autonomously in a host cell, and which is characterized by one or a small number of restriction endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without loss of an essential biological function of the vector, and into which DNA may be spliced in order to bring about its replication and cloning. The cloning vector may further contain a marker suitable for use in the identification of cells transformed with the cloning vector. Markers, for example, are tetracycline resistance or ampicillin resistance.

20 Recombinant host. Any prokaryotic or eukaryotic microorganism which contains the desired cloned genes in an expression vector, cloning vector or any DNA molecule. The term "recombinant host" is also meant to include those host

TO2907860

cells which have been genetically engineered to contain the desired gene on the host chromosome or genome.

Host. Any prokaryotic or eukaryotic microorganism that is the recipient of a replicable expression vector, cloning vector or any DNA molecule. The DNA molecule may contain, but is not limited to, a structural gene, a promoter and/or an origin of replication.

Promoter. A DNA sequence generally described as the 5' region of a gene, located proximal to the start codon. At the promoter region, transcription of an adjacent gene(s) is initiated.

Gene. A DNA sequence that contains information necessary for expression of a polypeptide or protein. It includes the promoter and the structural gene as well as other sequences involved in expression of the protein.

Structural gene. A DNA sequence that is transcribed into messenger RNA that is then translated into a sequence of amino acids characteristic of a specific polypeptide.

Operably linked. As used herein "operably linked" means that the promoter is positioned to control the initiation of expression of the polypeptide encoded by the structural gene.

Expression. Expression is the process by which a gene produces a polypeptide. It includes transcription of the gene into messenger RNA (mRNA) and the translation of such mRNA into polypeptide(s).

Substantially Pure. As used herein "substantially pure" means that the desired purified protein is essentially free from contaminating cellular contaminants which are associated with the desired protein in nature. Contaminating cellular components may include, but are not limited to, phosphatases, exonucleases, endonucleases or undesirable DNA polymerase enzymes.

Primer. As used herein "primer" refers to a single-stranded oligonucleotide that is extended by covalent bonding of nucleotide monomers during amplification or polymerization of a nucleic acid molecule. Minisatellite

primers used for the amplification of minisatellite dimer, trimer, tetramer, etc., sequences are well-known in the art.

5 **Template.** The term "template" as used herein refers to a double-stranded or single-stranded nucleic acid molecule which is to be amplified, synthesized or sequenced. In the case of a double-stranded DNA molecule, denaturation of its strands to form a first and a second strand is performed before these molecules may be amplified, synthesized or sequenced. A primer, complementary to a portion of a template is hybridized under appropriate conditions and the polymerase of the invention may then synthesize a molecule complementary to said template or a portion thereof. The newly synthesized molecule, according to the invention, may be equal or shorter in length than the original template. Mismatch incorporation or strand slippage during the synthesis or extension of the newly synthesized molecule may result in one or a number of mismatched base pairs. Thus, the synthesized molecule need not be exactly complementary to the template.

10 **Incorporating.** The term "incorporating" as used herein means becoming a part of a nucleic acid (e.g., DNA) molecule or primer.

15 **Amplification.** As used herein "amplification" refers to any *in vitro* method for increasing the number of copies of a nucleotide sequence with the use of a DNA polymerase. Nucleic acid amplification results in the incorporation of nucleotides into a DNA molecule or primer thereby forming a new DNA molecule complementary to a DNA template. The formed DNA molecule and its template can be used as templates to synthesize additional DNA molecules. As used herein, one amplification reaction may consist of many rounds of DNA replication. DNA amplification reactions include, for example, polymerase chain reactions (PCR). One PCR reaction may consist of 5 to 100 "cycles" of denaturation and synthesis of a DNA molecule.

20 **Oligonucleotide.** "Oligonucleotide" refers to a synthetic or natural molecule comprising a covalently linked sequence of nucleotides which are joined

by a phosphodiester bond between the 3' position of the pentose of one nucleotide and the 5' position of the pentose of the adjacent nucleotide.

Nucleotide. As used herein "nucleotide" refers to a base-sugar-phosphate combination. Nucleotides are monomeric units of a nucleic acid sequence (DNA and RNA). The term nucleotide includes deoxyribonucleoside triphosphates such as dATP, dCTP, dITP, dUTP, dGTP, dTTP, or derivatives thereof. Such derivatives include, for example, [α S]dATP, 7-deaza-dGTP and 7-deaza-dATP. The term nucleotide as used herein also refers to dideoxyribonucleoside triphosphates (ddNTPs) and their derivatives. Illustrated examples of dideoxyribonucleoside triphosphates include, but are not limited to, ddATP, ddCTP, ddGTP, ddITP, and ddTTP. According to the present invention, a "nucleotide" may be unlabeled or detectably labeled by well known techniques. Detectable labels include, for example, radioactive isotopes, fluorescent labels, chemiluminescent labels, bioluminescent labels and enzyme labels.

Thermostable. As used herein "thermostable" refers to a polymerase which is resistant to inactivation by heat. DNA polymerases synthesize the formation of a DNA molecule complementary to a single-stranded DNA template by extending a primer in the 5'-to-3' direction. This activity for mesophilic DNA polymerases may be inactivated by heat treatment. For example, T5 DNA polymerase activity is totally inactivated by exposing the enzyme to a temperature of 90°C for 30 seconds. As used herein, a thermostable polymerase activity is more resistant to heat inactivation than a mesophilic polymerase. However, a thermostable polymerase does not mean to refer to an enzyme which is totally resistant to heat inactivation and thus heat treatment may reduce the polymerase activity to some extent. A thermostable polymerase typically will also have a higher optimum temperature than mesophilic polymerases.

Hybridization. The terms "hybridization" and "hybridizing" refers to the pairing of two complementary single-stranded nucleic acid molecules (RNA and/or DNA) to give a double-stranded molecule. As used herein, two nucleic acid molecules may be hybridized, although the base pairing is not completely

10/290-20076860

complementary. Accordingly, mismatched bases do not prevent hybridization of two nucleic acid molecules provided that appropriate conditions, well known in the art, are used. In the present invention, the term "hybridization" refers particularly to hybridization of an oligonucleotide to a template molecule.

5 **3'-5' Exonuclease Activity.** "3'-5' exonuclease activity" is an enzymatic activity well known to the art. This activity is often associated with DNA polymerases, and is thought to be involved in a DNA replication "editing" or correction mechanism.

10 A "DNA polymerase substantially reduced in 3'-5' exonuclease activity" (which may also be represented as "3'-exo-") is defined herein as either (1) a mutated DNA polymerase that has about or less than 10%, or preferably about or less than 1%, of the 3'-5' exonuclease activity of the corresponding unmutated, wildtype enzyme, or (2) a DNA polymerase having a 3'-5' exonuclease specific activity which is less than about 1 unit/mg protein, or preferably about or less than 0.1 units/mg protein. A unit of activity of 3'-5' exonuclease is defined as the amount of activity that solubilizes 10 nmoles of substrate ends in 60 min. at 37°C, assayed as described in the "BRL 1989 Catalogue & Reference Guide", page 5, with *Hha*I fragments of *lambda* DNA 3'-end labeled with [³H]dTTP by terminal deoxynucleotidyl transferase (TdT). Protein is measured by the method of Bradford, *Anal. Biochem.* 72:248 (1976). As a means of comparison, natural, wildtype T5-DNA polymerase (DNAP) or T5-DNAP encoded by pTTQ19-T5-2 has a specific activity of about 10 units/mg protein while the DNA polymerase encoded by pTTQ19-T5-2(exo') (U.S. Patent No. 5,270,179) has a specific activity of about 0.0001 units/mg protein, or 0.001% of the specific activity of the unmodified enzyme, a 10²-fold reduction.

15 **5'-3' Exonuclease Activity.** "5'-3' exonuclease activity" is also an enzymatic activity well known in the art. This activity is often associated with DNA polymerases, such as *E. coli* PolI and PolIII.

20 A "DNA polymerase substantially reduced in 5'-3' exonuclease activity" (which may also be represented as "5'-exo-") is defined herein as either (1) a

TO23901235768990

5 mutated DNA polymerase that has about or less than 10%, or preferably about or less than 1%, of the 5'-3' exonuclease activity of the corresponding unmutated, wildtype enzyme, or (2) a DNA polymerase having 5'-3' exonuclease specific activity which is less than about 1 unit/mg protein, or preferably about or less than 0.1 units/mg protein.

10 Both of the 3'-5' and 5'-3' exonuclease activities can be observed on sequencing gels. Active 5'-3' exonuclease activity will produce nonspecific ladders in a sequencing gel by removing nucleotides from the 5'-end of the growing primers. 3'-5' exonuclease activity can be measured by following the degradation of radiolabeled primers in a sequencing gel. Thus, the relative amounts of these activities, e.g. by comparing wildtype and mutant polymerases, can be determined with no more than routine experimentation.

15 **Minisatellite DNA.** As used herein, the term "minisatellite DNA" refers to a DNA fragment comprising a short stretch of tandemly repetitive nucleotide sequence. *In vivo*, minisatellite DNA fragments are found interspersed throughout the genomes of most eukaryotic organisms thus far examined. These repeating sequences appear in tandem and often in variable numbers within the genome; thus, the terms "short tandem repeats" ("STRs") or "variable numbers of tandem repeats" ("VNTRs") may be used synonymously when referring to these regions.

20 Minisatellite DNA fragments are typically about 9 bases to about 60 bases in length and are repeated about 20-50 times at a typical locus in a eukaryotic genome.

25 **Microsatellite DNA.** As used herein, the term "microsatellite DNA" refers to DNA fragments which are typically of a repeat unit size of about 1-6 bases in length. The most prevalent of these microsatellite DNA fragments in the human genome is the dinucleotide repeat (dC-dA)_n•(dG-dT)_n (where n is the number of repetitions in a given stretch of nucleotides). The terms "STRs" and "VNTRs" may also be used synonymously to denote these structures.

30 **Non-templated 3' Terminal Nucleotide Addition.** As used herein, the term "non-templated 3' terminal nucleotide addition" or "extranucleotide addition"

TO/390-25216860

means the propensity of an enzyme such as a DNA polymerase to incorporate one or more additional nucleotides, which are not found in the template strand at the 3' terminus of a newly synthesized nucleic acid molecule in a synthesis or amplification reaction, such as PCR. As a result of non-templated 3' terminal nucleotide addition, the synthesized or amplification products (*i.e.*, the newly synthesized DNA strand) will be longer by one or more nucleotides than is the template, in such a fashion that if the template is "n" nucleotides in length, the synthesis or amplification products will be "n+1," "n+2," "n+3," etc., nucleotides in length. A "polymerase substantially reduced in the ability to add one or more non-templated nucleotides to the 3' terminus of a nucleic acid molecule" is defined herein as a DNA polymerase, which when it has no 3' exonuclease activity or has substantially reduced 3' exonuclease activity, it will produce a collection of amplification products in which less than about 50%, preferably less than about 30%, more preferably less than about 20%, still more preferably less than about 10%, still more preferably less than about 5%, and most preferably less than about 1% of the amplification products contain one or more non-templated nucleotides at their 3' termini compared to amplification products produced by *Taq* DNA polymerase assayed under the same conditions. Preferably, the conditions used for assaying 3' non-templated nucleotide addition is performed such that less than 100% of the amplification products of *Taq* DNA polymerase exhibits 3' non-templated nucleotide addition. Included in this definition are those polymerases that satisfy this definition for any primer set used. Thus, if the use of any primer set provides the indicated reduction of 3' non-templated nucleotide addition, the polymerase is said to be substantially reduced in the ability to add one or more non-templated nucleotides to the 3' terminus of a nucleic acid molecule.

When referring to polymerases which have been mutated or modified to reduce or eliminate 3' non-templated nucleotide addition, the mutated or modified polymerase is said to be "reduced in the ability to add one or more non-templated nucleotides to the 3' terminus of a nucleic acid molecule" when the polymerase has a lower or reduced or eliminated ability to add non-templated 3' nucleotides

10/29/2016 3:30:00 PM

5
10
15
20
25
30

compared to the corresponding unmutated, unmodified or wildtype polymerase. For example, when testing the affect of a point mutation in the O-helix of a polymerase on non-templated nucleotide addition, the polymerase unmodified in the same position of the O-helix is preferably used for comparison purposes. Such 5 mutated or modified polymerases are said to "substantially reduced in the ability to add one or more non-templated nucleotides to the 3' terminus of a nucleic acid molecule" if the mutated or modified polymerase has less than about 50%, preferably less than about 30%, more preferably less than about 20%, still more preferably less than about 10%, still more preferably less than about 5%, and most preferably less than about 1% of the activity for adding non-templated 3' terminal nucleotides compared to the corresponding unmutated, unmodified or wildtype polymerase. Preferably, the conditions used for assaying 3' non-templated nucleotide addition is performed such that less than 100% of the amplification 10 products produced by the unmutated, unmodified or wildtype polymerase control exhibits 3' non-templated nucleotide addition. Included in this definition are those 15 mutant or modified polymerases that satisfy this definition for any primer set tested.

The ability of a polymerase to add a non-templated 3' terminal nucleotide to the growing strand may be assessed by a variety of techniques, most preferably 20 by gel electrophoresis of the synthesized or amplification products for a direct size comparison and by comparison to markers of known size (see Figures 6-13).

Other terms used in the fields of recombinant DNA technology and molecular and cell biology as used herein will be generally understood by one of ordinary skill in the applicable arts.

25

Sources of Polymerases

The methods of the present invention rely on the use of polymerases (thermostable or mesophilic DNA or RNA polymerases) reduced, substantially reduced or eliminated in the ability to add one or more non-templated 3' terminal nucleotide to a growing nucleic acid strand. These thermostable DNA

PCT/US2003/035960

polymerases may be obtained from any strain of any thermophilic microorganism, including but not limited to strains of *Thermus aquaticus* (*Taq* polymerase; see U.S. Patent Nos. 4,889,818 and 4,965,188), *Thermus thermophilus* (*Tth* polymerase), *Thermococcus litoralis* (*Tli* or *VENT*TM polymerase), *Pyrococcus furiosus* (*Pfu* or *DEEPVENT*TM polymerase), *Pyrococcus woosii* (*Pwo* polymerase) and other *Pyrococcus* species, *Bacillus stearothermophilus* (*Bst* polymerase), *Sulfolobus acidocaldarius* (*Sac* polymerase), *Thermoplasma acidophilum* (*Tac* polymerase), *Bacillus caldophilus* (*Bca* polymerase), *Thermus flavus* (*Tfl/Tub* polymerase), *Thermus ruber* (*Tru* polymerase), *Thermus brockianus* (*DYNAZYME*TM polymerase), *Thermotoga neapolitana* (*The* polymerase; see WO 96/10640 and WO96/41014), *Thermotoga maritima* (*Tma* polymerase; see U.S. Patent No. 5,374,553) and other species of the *Thermotoga* genus (*Tsp* polymerase) and *Methanobacterium thermoautotrophicum* (*Mth* polymerase). Mesophilic DNA polymerases of interest in the invention include but are not limited to T7 DNA polymerases, T5 DNA polymerase, DNA polymerase III, Klenow fragment DNA polymerase and mutants, fragments or derivatives thereof. RNA polymerases such as T3, T5, SP6 and mutants, variants and derivatives thereof may also be used in accordance with the invention. Polymerases having reduced or substantially reduced ability to add a non-templated 3' nucleotide to a growing nucleic acid strand may be wildtype polymerases, or may be made by mutating such wildtype polymerases by standard techniques (for example, by generating point mutations, insertions, deletions, etc., in the wildtype gene or protein). Polymerases that are reduced or substantially reduced in the ability to add a non-templated 3' nucleotide to a growing strand may be identified by assaying the synthesized products (e.g. PCR products) formed by such enzymes, as is well-known in the art and as generally described below in the Examples.

The nucleic acid polymerases used in the present invention may be mesophilic or thermophilic, and are preferably thermophilic. Preferred mesophilic DNA polymerases include T7 DNA polymerase, T5 DNA polymerase, Klenow

100-1000-1000-1000

5

10

15

20

25

30

fragment DNA polymerase, DNA polymerase III and the like. Preferred thermostable DNA polymerases that may be used in the methods of the invention include *Taq*, *Tne*, *Tma*, *Pfu*, *Tfl*, *Tth*, Stoffel fragment, VENT™ and DEEPVENT™ DNA polymerases, and mutants, variants and derivatives thereof (U.S. Patent No. 5,436,149; U.S. Patent 4,889,818; U.S. Patent 4,965,188; U.S. Patent 5,079,352; U.S. Patent 5,614,365; U.S. Patent 5,374,553; U.S. Patent 5,270,179; U.S. Patent 5,047,342; U.S. Patent No. 5,512,462; WO 92/06188; WO 92/06200; WO 96/10640; Barnes, W.M., *Gene* 112:29-35 (1992); Lawyer, F.C., *et al.*, *PCR Meth. Appl.* 2:275-287 (1993); Flaman, J.-M., *et al.*, *Nucl. Acids Res.* 22(15):3259-3260 (1994)). For amplification of long nucleic acid molecules (e.g., nucleic acid molecules longer than about 3-5 Kb in length), at least two DNA polymerases (one substantially lacking 3' exonuclease activity and the other having 3' exonuclease activity) are typically used. See U.S. Patent No. 5,436,149; U.S. Patent No. 5,512,462; Farnes, W.M., *Gene* 112:29-35 (1992); and copending U.S. Patent Application No. 08/689,814, filed February 14, 1997, the disclosures of which are incorporated herein in their entireties. Examples of DNA polymerases substantially lacking in 3' exonuclease activity include, but are not limited to, *Taq*, *Tne*(exo⁻), *Tma*(exo⁻), *Pfu* (exo⁻), *Pwo*(exo⁻) and *Tth* DNA polymerases, and mutants, variants and derivatives thereof.

Polypeptides having nucleic acid polymerase activity are preferably used in the present methods at a final concentration in solution of about 0.1-200 units per milliliter, about 0.1-50 units per milliliter, about 0.1-40 units per milliliter, about 0.1-3.6 units per milliliter, about 0.1-34 units per milliliter, about 0.1-32 units per milliliter, about 0.1-30 units per milliliter, or about 0.1-20 units per milliliter, and most preferably at a concentration of about 20-40 units per milliliter. Of course, other suitable concentrations of nucleic acid polymerases suitable for use in the invention will be apparent to one or ordinary skill in the art.

In a preferred aspect of the invention, polymerases of the invention and preferably the mutant or modified polymerases of the invention are made by

102598122-001

recombinant techniques. A number of cloned polymerase genes are available or may be obtained using standard recombinant techniques.

To clone a gene encoding a polymerase, which may be modified in accordance with the invention, isolated DNA which contains the polymerase gene is used to construct a recombinant library in a vector. Any vector, well known in the art, can be used to clone the DNA polymerase of interest. However, the vector used must be compatible with the host in which the recombinant DNA library will be transformed.

Prokaryotic vectors for constructing the plasmid library include plasmids such as those capable of replication in *E. coli* such as, for example, pBR322, ColE1, pSC101, pUC-vectors (pUC18, pUC19, etc.: In: *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1982); and Sambrook *et al.*, In: *Molecular Cloning A Laboratory Manual* (2d ed.) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989)). *Bacillus* plasmids include pC194, pC221, pC217, etc. Such plasmids are disclosed by Glyczan, T. In: *The Molecular Biology Bacilli*, Academic Press, York (1982), 307-329. Suitable *Streptomyces* plasmids include pIJ101 (Kendall *et al.*, *J. Bacteriol.* 169:4177-4183 (1987)). *Pseudomonas* plasmids are reviewed by John *et al.*, (*Rad. Insec. Dis.* 8:693-704 (1986)), and Igaki, (*Jpn. J. Bacteriol.* 33:729-742 (1978)). Broad-host range plasmids or cosmids, such as pCP13 (Darzins and Chakrabarbarby, *J. Bacteriol.* 159:9-18, 1984) can also be used for the present invention. The preferred vectors for cloning the genes of the present invention are prokaryotic vectors. Preferably, pCP13 and pUC vectors are used to clone the genes of the present invention.

The preferred host for cloning the polymerase genes of interest is a prokaryotic host. The most preferred prokaryotic host is *E. coli*. However, the desired polymerase genes of the present invention may be cloned in other prokaryotic hosts including, but not limited to, *Escherichia*, *Bacillus*, *Streptomyces*, *Pseudomonas*, *Salmonella*, *Serratia*, and *Proteus*. Bacterial hosts

TO/290-32246360

of particular interest include *E. coli* DH10B, which may be obtained from Life Technologies, Inc. (LTI) (Rockville, MD).

Eukaryotic hosts for cloning and expression of the polymerases of interest include yeast, fungi, and mammalian cells. Expression of the desired polymerase in such eukaryotic cells may require the use of eukaryotic regulatory regions which include eukaryotic promoters. Cloning and expressing the polymerase gene in eukaryotic cells may be accomplished by well known techniques using well known eukaryotic vector systems.

Once a DNA library has been constructed in a particular vector, an appropriate host is transformed by well known techniques. Transformed colonies are preferably plated at a density of approximately 200-300 colonies per petri dish. For thermostable polymerase selection, colonies are then screened for the expression of a heat stable DNA polymerase by transferring transformed *E. coli* colonies to nitrocellulose membranes. After the transferred cells are grown on nitrocellulose (approximately 12 hours), the cells are lysed by standard techniques, and the membranes are then treated at 95°C for 5 minutes to inactivate the endogenous *E. coli* enzyme. Other temperatures may be used to inactivate the host polymerases depending on the host used and the temperature stability of the polymerase to be cloned. Stable polymerase activity is then detected by assaying for the presence of polymerase activity using well known techniques (see, e.g., Sagner *et al.*, *Gene* 97:119-123 (1991), which is hereby incorporated by reference in its entirety). The gene encoding a polymerase of the present invention can be cloned using the procedure described by Sanger *et al.*, *supra*. Other techniques for selecting cloned polymerases in accordance with the present invention will be well-known to those of ordinary skill in the art.

Modifications or Mutations of Polymerases

In accordance with the invention, the nucleotide binding domain of the polymerase of interest is modified or mutated in such a way as to produce a mutated or modified polymerase having reduced, substantially reduced or

10/22/2012 10:36:00 AM

eliminated activity for adding non-templated 3' nucleotides. The O-helix region typically defines the nucleotide binding domain of DNA polymerases. The O-helix may be defined as RXXXXXXXFXXXXY (SEQ ID NO:11), wherein X may be any amino acid. One or more mutations or combinations of mutations may be made in the O-helix of any polymerase in order to reduce or eliminate non-templated 3' nucleotide addition in accordance with the invention. Such mutations include point mutation, frame-shift mutations, deletions and insertions. Preferably, one or more point mutations, resulting in one or more amino acid substitutions, are used to produce polymerases having such activity. Such mutations may be made by a number of methods that will be familiar to one of ordinary skill, including but not limited to site-directed mutagenesis. In a preferred aspect of the invention, one or more mutations at positions R, K, F, and/or Y in the polymerase O-helix may be made to produce a polymerase having the desired activity. Most preferably, one or more mutations at position R and/or F and/or K and/or Y within the O-helix results in polymerases having reduced, substantially reduced or eliminated activity for adding non-templated 3' nucleotides. In the preferred aspect, amino acid substitutions are made at position R and/or F and/or K and/or Y (or combinations thereof). Thus, R (Arg) and/or F (Phe) and/or K (Lys) may be substituted with any other amino acid including Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val. Preferably, R (Arg) is substituted with amino acids Lys, Tyr, Leu, His, Gln, Met, or Asn. F (Phe) is preferably substituted with amino acids Tyr, Ala, Leu, Thr, and Ser. K (Lys) is preferably substituted with amino acids Arg, Tyr, Leu, His, Gln, Met or Asn, and more preferably with Arg or His. Y (Tyr) is preferably substituted with amino acids Lys, Arg, Ala, Thr, Phe, Leu, His, Gln, Met, or Asn. Positions corresponding to R, K, F and Y for RNA polymerases may also be determined by comparing nucleotide and/or amino acid sequences with those of DNA polymerases, to determine homologies therebetween. Corresponding mutations or modification may then be made to produce the desired result in any RNA polymerase.

The O-helix has been identified and defined for a number of polymerases and may be readily identified for other polymerases by one with skill in the art. Thus, given the defined O-helix region and the methods and assays described herein, one with skill in the art can make one or a number of modifications which would result in polymerases having reduced, substantially reduced or eliminated activity for adding non-templated 3' nucleotides. Accordingly, the invention relates to methods for producing such polymerases having modifications in the O-helix domain resulting in reduction, substantial reduction or elimination of activity for adding non-templated 3' nucleotides, methods for producing nucleic acid molecules encoding such polymerases, and polymerases and nucleic acid molecules produced by such methods.

The following table illustrates identified O-helix regions for known polymerases.

Polymerase	O-Helix Region	SEQ ID NO.
PoII	754 RRSAKAINFGGLIYG	12
<i>Tag</i>	659 RRAAKTINFGVLYG	13
T7	518 RDNAKTFIYGFLYG	14
<i>Tne</i>	722 RRVGKVMVNFSIIYG	15
T5	588 RQAAKAITFGILYG	16
<i>Tma</i>	722 RRAGKVMVNFSIIYG	17

Thus, in accordance with a preferred aspect of the invention, corresponding mutations in the R and/or F and/or K positions of the O-helix can be made for the following enzymes based on the tables below.

Polymerase	Mutation Position
PolI	Arg ⁷⁵⁴
T5	Arg ⁵⁸⁸
T7	Arg ⁵¹⁸
5 <i>Taq</i>	Arg ⁶⁵⁹
<i>Tne</i>	Arg ⁷²²
<i>Tma</i>	Arg ⁷²²
<i>Bca</i>	Arg ⁷⁰⁵
<i>Bst</i>	Arg ⁷⁰²
<i>Tth</i>	Arg ⁶⁶¹

Polymerase	Mutation Position
PolI	Phe ⁷⁶²
T5	Phe ⁵⁹⁶
T7	Phe ⁵²⁸
15 <i>Taq</i>	Phe ⁶⁶⁷
<i>Tne</i>	Phe ⁷³⁰
<i>Tma</i>	Phe ⁷³⁰
<i>Bca</i>	Phe ⁷¹³
<i>Bst</i>	Phe ⁷¹⁰
20 <i>Tth</i>	Phe ⁶⁶⁹

Polymerase	Mutation Position
PolI	Lys ⁷⁵⁸
T5	Lys ⁵⁹²
T7	Lys ⁵²²
25 <i>Taq</i>	Lys ⁶⁶³
<i>Tne</i>	Lys ⁷²⁶
<i>Tma</i>	Lys ⁷²⁶
<i>Bca</i>	Lys ⁷⁰⁷
<i>Bst</i>	Lys ⁷⁰⁶
30 <i>Tth</i>	Lys ⁶⁶⁵

The mutation position of Arg⁷⁰⁵ for *Bca* is based on the sequence information in GenBank. It should be noted, however, that according to the sequence described by Vemori *et al.* *J. Biochem. (Japan)* 113:401-410 (1993), the position of Arg in *Bca* is 703.

5 **Additional Modifications or Mutations of Polymerases**

In accordance with the invention, in addition to the mutations or modifications described above, one or more additional mutations or modifications (or combinations thereof) may be made to the polymerases of interest. Mutations or modifications of particular interest include those modifications of mutations which (1) reduce or eliminate 3' to 5' exonuclease activity; and (2) reduce or eliminate 5' to 3' exonuclease activity.

If the DNA polymerase has 3'-to-5' exonuclease activity, this activity may be reduced, substantially reduced, or eliminated by mutating the polymerase gene. Such mutations include point mutations, frame shift mutations, deletions and insertions. Preferably, the region of the gene encoding the 3'-to-5' exonuclease activity is mutated or deleted using techniques well known in the art (Sambrook *et al.*, (1989) in: *Molecular Cloning, A Laboratory Manual* (2nd Ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

The 3'-to-5' exonuclease activity can be reduced or impaired by creating site specific mutants within the 3'→5' exonuclease domain. *See infra*. In a specific embodiment of the invention Asp³²³ of *Tne* DNA polymerase is changed to any amino acid, preferably to Ala³²³ to substantially reduce 3'→5' exonuclease activity. In another specific embodiment of the invention, Asp³²³ of *Tma* may be changed to any other amino acid, preferably to Ala to substantially reduce 3'→5' exonuclease activity. The following represents a domain of interest for a number of polymerases for preparing 3'→5' exonuclease mutants.

102/2901-30016860

<i>Tne</i>	318	PSFALDLETSS	328	(SEQ ID NO:18)
Pol I	350	PVFAFDTETDS	360	(SEQ ID NO:19)
T5	159	GPVAFDSETSA	169	(SEQ ID NO:20)
T7	1	MIVSDIEANA	10	(SEQ ID NO:21)

5 Mutations, such as insertions, deletions and substitutions within the above domain can result in substantially reduced 3'→5' exonuclease activity. By way of example, Asp³⁵⁵ (PolI), Asp¹⁶⁴ (T5), and Asp⁵ (T7) may be substituted with any amino acid to substantially reduce 3'→5' exonuclease activity. For example, Asp at these positions may be substituted with Ala.

10 The 5'→3' exonuclease activity of the polymerases can be reduced, substantially reduced or eliminated by mutating the polymerase gene or by deleting the 5' to 3' exonuclease domain. Such mutations include point mutations, frame shift mutations, deletions, and insertions. Preferably, the region of the gene encoding the 5'→3' exonuclease activity is deleted using techniques well known in the art. In embodiments of this invention, any one of six conserved amino acids that are associated with the 5'→3' exonuclease activity can be mutated. Examples of these conserved amino acids with respect to *Tne* DNA polymerase include Asp⁸, Glu¹¹², Asp¹¹⁴, Asp¹¹⁵, Asp¹³⁷, and Asp¹³⁹. Other possible sites for mutation are Gly¹⁰², Gly¹⁸⁷ and Gly¹⁹⁵.

15 20 Corresponding amino acid to target for other polymerases to reduce or eliminate 5'→3' exonuclease activity as follows:

E. coli polII: Asp¹³, Glu¹¹³, Asp¹¹⁵, Asp¹¹⁶, Asp¹³⁸, and Asp¹⁴⁰.

Taq pol: Asp¹⁸, Glu¹¹⁷, Asp¹¹⁹, Asp¹²⁰, Asp¹⁴², and Asp¹⁴⁴.

Tma pol: Asp⁸, Glu¹¹², Asp¹¹⁴, Asp¹¹⁵, Asp¹³⁷, and Asp¹³⁹.

25 Amino acid residues of *Taq* DNA polymerase are as numbered in U.S. 5,079,352. Amino acid residues of *Thermotoga maritima* (*Tma*) DNA polymerase are numbered as in U.S. Patent No. 5,374,553.

Examples of other amino acids which may be targeted for other polymerases to reduce 5'→3' exonuclease activity include:

Enzyme or source	Mutation positions
<i>Streptococcus pneumoniae</i>	Asp ¹⁰ , Glu ¹¹⁴ , Asp ¹¹⁶ , Asp ¹¹⁷ , Asp ¹³⁹ , Asp ¹⁴¹
<i>Thermus flavus</i>	Asp ¹⁷ , Glu ¹¹⁶ , Asp ¹¹⁸ , Asp ¹¹⁹ , Asp ¹⁴¹ , Asp ¹⁴³
<i>Thermus thermophilus</i>	Asp ¹⁸ , Glu ¹¹⁸ , Asp ¹²⁰ , Asp ¹²¹ , Asp ¹⁴³ , Asp ¹⁴⁵
<i>Deinococcus radiodurans</i>	Asp ¹⁸ , Glu ¹¹⁷ , Asp ¹¹⁹ , Asp ¹²⁰ , Asp ¹⁴² , Asp ¹⁴⁴
<i>Bacillus caldotenax</i>	Asp ⁹ , Glu ¹⁰⁹ , Asp ¹¹¹ , Asp ¹¹² , Asp ¹³⁴ , Asp ¹³⁶

Coordinates of *S. pneumoniae*, *T. flavus*, *D. radiodurans*, *B. caldotenax* were obtained from Gutman and Minton. Coordinates of *T. thermophilus* were obtained from International Patent No. WO 92/06200.

Typically, the mutant polymerases of the invention can be affected by substitution of amino acids typically which have different properties. For example, an acidic amino acid such as Asp may be changed to a basic, neutral or polar but uncharged amino acid such as Lys, Arg, His (basic); Ala, Val, Leu, Ile, Pro, Met, Phe, Trp (neutral); or Gly, Ser, Thr, Cys, Tyr, Asn or Gln (polar but uncharged). Glu may be changed to Asp, Ala, Val Leu, Ile, Pro, Met, Phe, Trp, Gly, Ser, Thr, Cys, Tyr, Asn or Gln.

Preferably, oligonucleotide directed mutagenesis is used to create the mutant polymerases which allows for all possible classes of base pair changes at any determined site along the encoding DNA molecule. In general, this technique involves annealing a oligonucleotide complementary (except for one or more mismatches) to a single stranded nucleotide sequence coding for the DNA polymerase of interest. The mismatched oligonucleotide is then extended by DNA polymerase, generating a double stranded DNA molecule which contains the desired change in the sequence on one strand. The changes in sequence can of course result in the deletion, substitution, or insertion of an amino acid. The double stranded polynucleotide can then be inserted into an appropriate expression

vector, and a mutant polypeptide can thus be produced. The above-described oligonucleotide directed mutagenesis can of course be carried out via PCR.

Enhancing Expression of Polymerases

To optimize expression of the polymerases of the present invention, 5 inducible or constitutive promoters are well known and may be used to express high levels of a polymerase structural gene in a recombinant host. Similarly, high copy number vectors, well known in the art, may be used to achieve high levels of expression. Vectors having an inducible high copy number may also be useful to enhance expression of the polymerases of the invention in a recombinant host.

10 To express the desired structural gene in a prokaryotic cell (such as, *E. coli*, *B. subtilis*, *Pseudomonas*, etc.), it is necessary to operably link the desired structural gene to a functional prokaryotic promoter. However, the natural promoter of the polymerase gene may function in prokaryotic hosts allowing expression of the polymerase gene. Thus, the natural promoter or other

15 promoters may be used to express the polymerase gene. Such other promoters may be used to enhance expression and may either be constitutive or regulatable (i.e., inducible or derepressible) promoters. Examples of constitutive promoters include the *int* promoter of bacteriophage λ , and the *bla* promoter of the β -lactamase gene of pBR322. Examples of inducible prokaryotic promoters include the major right and left promoters of bacteriophage λ (P_R and P_L), *trp*,

20 *recA*, *lacZ*, *lacI*, *tet*, *gal*, *trc*, and *tac* promoters of *E. coli*. The *B. subtilis* promoters include α -amylase (Ulmanen *et al.*, *J. Bacteriol.* 162:176-182 (1985)) and *Bacillus* bacteriophage promoters (Gryczan, T., In: *The Molecular Biology Of Bacilli*, Academic Press, New York (1982)). *Streptomyces* promoters are described by Ward *et al.*, *Mol. Gen. Genet.* 203:468478 (1986)).

25 Prokaryotic promoters are also reviewed by Glick, *J. Ind. Microbiol.* 1:277-282 (1987); Cenatiempo, Y., *Biochimie* 68:505-516 (1986); and Gottesman, *Ann. Rev. Genet.* 18:415-442 (1984). Expression in a prokaryotic cell also requires the presence of a ribosomal binding site upstream of the gene-encoding sequence. Such ribosomal

10239032335860

binding sites are disclosed, for example, by Gold *et al.*, *Ann. Rev. Microbiol.* 35:365404 (1981).

To enhance the expression of polymerases of the invention in a eukaryotic cell, well known eukaryotic promoters and hosts may be used. Preferably, however, enhanced expression of the polymerases is accomplished in a prokaryotic host. The preferred prokaryotic host for overexpressing the polymerases of the invention is *E. coli*.

Isolation and Purification of Polymerases

The enzyme(s) of the present invention is preferably produced by fermentation of the recombinant host containing and expressing the desired polymerase gene. However, the polymerases of the present invention may be isolated from any strain which produces the polymerase of the present invention. Fragments of the polymerase are also included in the present invention. Such fragments include proteolytic fragments and fragments having polymerase activity.

Any nutrient that can be assimilated by a host containing the polymerase gene may be added to the culture medium. Optimal culture conditions should be selected case by case according to the strain used and the composition of the culture medium. Antibiotics may also be added to the growth media to insure maintenance of vector DNA containing the desired gene to be expressed. Media formulations have been described in DSM or ATCC Catalogs and Sambrook *et al.*, In: *Molecular Cloning, a Laboratory Manual* (2nd ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).

Host cells producing the polymerases of this invention can be separated from liquid culture, for example, by centrifugation. In general, the collected microbial cells are dispersed in a suitable buffer, and then broken down by ultrasonic treatment or by other well known procedures to allow extraction of the enzymes by the buffer solution. After removal of cell debris by ultracentrifugation or centrifugation, the polymerase can be purified by standard protein purification techniques such as extraction, precipitation, chromatography, affinity

10 15 20 25
TD/280-2806960

chromatography, electrophoresis or the like. Assays to detect the presence of the polymerase during purification are well known in the art and can be used during conventional biochemical purification methods to determine the presence of these enzymes.

5

***Thermotoga* Polymerases**

Thermotoga polymerases for use in the present invention are obtained from any strain of *Thermotoga* species, more preferably from a strain of *Thermotoga neapolitana* (WO 96/10640 or WO96/41014) or *Thermotoga maritima* (U.S. Patent No. 5,374,553). Enzymes suitable for use in the present invention from these more preferred sources are the wildtype DNA polymerases (*Tne* from *T. neapolitana*; *Tma* from *T. maritima*), or mutants or derivatives thereof.

The present invention provides isolated nucleic acid molecules encoding preferred mutant *Tne* DNA polymerases, mutant *Tne* DNA polymerases encoded by such isolated nucleic acid molecules, and specific mutant *Tne* DNA polymerase proteins. Most preferred are the wildtype *Tne* DNA polymerase (SEQ ID NOs:1,2), the wildtype *Tma* DNA polymerase (U.S. Patent No. 5,374,553), and the following mutants of *Tne* DNA polymerase: *Tne* N'Δ219, D323A (SEQ ID NO:3); *Tne* N'Δ283, D323A (SEQ ID NO:4); *Tne* N'Δ192, D323A (SEQ ID NO:5); *Tne* D137A, D323A (SEQ ID NO:6); *Tne* D8A, D323A (SEQ ID NO:7); *Tne* G195D, D323A (SEQ ID NO:8); *Tne* G37D, D323A (SEQ ID NO:9); *Tne* N'Δ283 (SEQ ID NO:10); *Tne* D137A, D323A, R722K; *Tne* D137A, D323A, R722Y; *Tne* D137A, D323A, R722L; *Tne* D137A, D323A, R722H; *Tne* D137A, D323A, R722Q; *Tne* D137A, D323A, F730Y; *Tne* D137A, D323A, K726R; *Tne* D137A, D323A, K726H; *Tne* D137A, D323A, R722K, F730Y; *Tne* D137A, D323A, R722K, K726R; *Tne* D137A, D323A, R722K, K726H; *Tne* D137A, D323A, R722H, F730Y; *Tne* D137A, D323A, R722H, K726R; *Tne* D137A, D323A, R722H, K726H; *Tne* D137A, D323A, R722Q, F730Y; *Tne* D137A, D323A, R722Q, K726R; *Tne* D137A, D323A, R722Q, K726H; *Tne* D137A, D323A, R722N, F730Y; *Tne* D137A, D323A, R722N, K726R;

10 102960226360

10

15

20

25

The D137A, D323A, R722N, K726H; *The* D137A, D323A, F730S; *The* N'D283, D323A, R722K/H/Q/N/Y/L; *The* N'D219, D323A, R722K; *The* N'D219, D323A, F730Y; *The* N'D219, D323A, K726R; *The* N'D219, D323A, K726H; *The* D137A, D323A, F730S, R722K/Y/Q/N/H/L, K726R/H; *The* D137A, D323A, F730T, R722K/Y/Q/N/H/L, K726R/H; *The* D137A, D323A, F730T; *The* F730S; *The* F730A; *The* K726R; *The* K726H; and *The* D137A, D323A, R722N. It will of course be understood by the skilled artisan that the designations of the above-described mutant polymerases indicate the position of the amino acid residue in the wildtype amino acid sequence (SEQ ID NO:2) that is being mutated, as well as to what residue the amino acid is being mutated. Thus, for example, "D137A" indicates that the Asp (D) residue at position 137 in SEQ ID NO:2 has been mutated to an Ala (A) residue, and, for example, "R722K/Y/Q/N/H/L" indicates that the Arg (R) residue at position 722 in SEQ ID NO:2 has been mutated to a Lys (K), Tyr (Y), Gln (Q), Asn (N), His (H) or Leu (L) residue. Mutant polymerases having one or more mutations or modifications corresponding to the *The* mutants of the invention are also contemplated by the invention.

The following chart indicates the nucleic acid sequences of the nucleic acid molecules encoding the above-described mutant *The* DNA polymerases (SEQ ID NOs:3-10), each with reference to the wildtype *The* DNA polymerase (SEQ ID NO:1):

10242901 20070605

5

10

15

20

5

09891332-062701

10

SEQ ID NO:	Deletion of SEQ ID NO: 1	Insertion	Substitution to SEQ ID NO: 1
3	Deletion of positions 1-657 from the 5'-end	ATG AGC TTC at the 5'-end	A replaces G at position 966; C replaces A at 968 and G replaces C at 969
4	Deletion of positions 1-849 from the 5'-end	None	A replaces G at 966; C replaces A at 968 and G replaces C at 969
5	Deletion of positions 1-576 from the 5'-end	ATG AAT TCG AGC TCG GTA CCC at the 5'-end	A replaces G at 966; C replaces A at 968 and G replaces C at 969; A replaces G at 584
6	None	None	A replaces G at 966; C replaces A at 968 and G replaces C at 969; C replaces T at 408 and C replaces A at 410
7	None	None	A replaces G at 966; C replaces A at 968 and G replaces C at 969; C replaces A at 23 and C replaces T at 24
8	None	None	A replaces G at 966; C replaces A at 968 and G replaces C at 969; T replaces C at 576 and A replaces G at 584
9	None	None	A replaces G at 966; C replaces A at 968 and G replaces C at 969; A replaces G at 110
10	Deletion of positions 1-849 from the 5'-end	None	None

Using these same approaches, the sequence guidance provided herein, and knowledge of appropriate nucleotide substitutions to be made to SEQ ID NO:1, one of ordinary skill can readily produce other nucleic acid molecules encoding mutant polymerases, such as those described in detail above, having the desired activity. In addition, other nucleic acid molecules which comprise a sequence

15

substantially different from those described above but which, due to the degeneracy of the genetic code, still encode a mutant *Tne* DNA polymerase having an amino acid sequence set forth above, are also encompassed by the present invention. Since the genetic code is well known in the art, it is routine for one of ordinary skill in the art to produce such mutants and degenerate variants without undue experimentation.

Each of these mutant *Tne* DNA polymerases are reduced or substantially reduced in the ability to add a non-templated 3' terminal nucleotide to the growing strand. These mutant *Tne* DNA polymerase proteins may be prepared by recombinant DNA techniques routine to one of ordinary skill. Preferably, such mutant *Tne* polymerases are prepared by inserting an isolated DNA molecule having a nucleotide sequence as described above for each individual mutant into a recombinant vector, inserting the vector into a host cell, preferably an *Escherichia coli* cell, and culturing the host cell under conditions favoring the production of the mutant *Tne* DNA polymerase. The mutant *Tne* polymerase is then isolated from the host cell according to standard protein purification techniques. Further guidance for the preparation and isolation of mutant DNA polymerases from thermostable microorganisms can be found, for example, in U.S. Patent No. 5,374,553, in co-pending U.S. Patent Application No. 08/689,818 of Deb K. Chatterjee and A. John Hughes, entitled "Cloned DNA Polymerases from *Thermotoga* and Mutants Therof," filed September 6, 1996, and in co-pending U.S. Patent Application No. 08/689,807 of Deb K. Chatterjee, entitled "Cloned DNA Polymerases from *Thermotoga* and Mutants Therof," filed September 6, 1996, the disclosures of all of which are incorporated herein in their entirety.

In the methods of the present invention, *Thermotoga* DNA polymerases substantially reduced in 3'-5' exonuclease activity (such as a *Tne* mutant having an amino acid sequence as set forth in any one of SEQ ID NOs:3-9), or *Thermotoga* DNA polymerases not substantially reduced in 3'-5' exonuclease activity (such as *Tne* DNA polymerase (SEQ ID NOs:1,2), *Tma* DNA polymerase (U.S. Patent No. 5,374,553), or the *Tne* mutant *Tne*N Δ 283 (SEQ ID NO:10)), may be used with

TO/380-22546860

similar results, since both types of *Thermotoga* DNA polymerase are substantially reduced in the ability to add a nontemplated 3' terminal nucleotide to a DNA template. Other thermostable DNA polymerases substantially reduced in 3'-5' exonuclease activity, such as *Taq*, VENT™(exo-), DEEPVENT™(exo-), 5 DtoK(exo-) and THERMOLASE™ *Tbr*, are not preferred for use in the present methods as they will add non-templated nucleotides to the 3' termini of the amplification products as described below. However, such thermostable polymerase can be made which have reduced, substantially reduced or eliminated activity to add 3' non-template nucleotides by mutating or modifying the polymerase in accordance with the invention. The preferred *Thermotoga* polymerases of the invention contain such mutations or modifications in their 10 O-helix.

15 The recombinant host comprising the gene encoding *Tne* DNA polymerase, *E. coli* DH10B(pUC-Tne), was deposited on September 30, 1994, with the Collection, Agricultural Research Culture Collection (NRRL), 1815 North University Street, Peoria, Illinois 61604 USA, as Deposit No. NRRL B-21238. The gene encoding *Tma* DNA polymerase has also been cloned and sequenced (U.S. Patent No. 5,374,553, which is expressly incorporated by reference herein in its entirety). Methods for preparing mutants and derivatives 20 of these *Tne* and *Tma* polymerases are well-known in the art, and are specifically described in co-pending U.S. Patent Application No. 08/689,818 of Deb K. Chatterjee and A. John Hughes, entitled "Cloned DNA Polymerases from *Thermotoga* and Mutants Therof," filed September 6, 1996, and co-pending U.S. Patent Application No. 08/689,807 of Deb K. Chatterjee, entitled "Cloned DNA 25 Polymerases from *Thermotoga* and Mutants Therof," filed September 6, 1996, the disclosures of which are incorporated herein in their entirety.

Advantages of Thermostable Polymerases

The use of thermostable polymerases (e.g. *Thermotoga* polymerases) or mutants or derivatives thereof in the methods of the present invention provide

TO/2001-20576890

several distinct advantages. These advantages are particularly apparent in the application of the present methods to analysis and typing of minisatellite, microsatellite and STR DNA regions.

With respect to traditional thermolabile DNA polymerases used in DNA amplification and sequencing, such as T4, T7 or *E. coli* Klenow fragment polymerases, thermostable polymerases such as *Thermotoga* DNA polymerases maintain their enzymatic activity in the multiple high-temperature cycles used in PCR and analogous automated amplification methodologies. It is therefore unnecessary to add fresh enzyme at the beginning of each amplification cycle when using thermostable polymerases, as must be done when thermolabile enzymes are used.

With respect to other thermostable enzymes, it has been unexpectedly discovered in the present invention (as described in more detail in the Examples below) that the use of *Tne* or *Tma* DNA polymerase mutants or derivatives thereof, does not result in the incorporation of non-templated 3' nucleotides into the newly synthesized DNA strands during DNA amplification reactions. This non-templated incorporation is a common problem when using certain other commonly employed thermostable enzymes, such as *Taq*, VENTTM(exo-), DEEPVENTTM(exo-), Dtok(exo-) and THERMOLASETM *Tbr*. It has also been unexpectedly discovered that mutants of these polymerases can be made to reduce or eliminate addition of non-templated 3' nucleotides. In particular, such mutations are preferably made within the O-helix of such polymerases.

Thus, the use of *Tne* or *Tma* DNA polymerases or mutants or derivatives thereof (or other mutant polymerases produced according to the invention) in amplifying and typing DNA sequences, particularly hypervariable DNA sequences such as minisatellite, microsatellite or STR regions, will allow a faithful amplification and resolution of polymorphisms in these regions. This faithful resolution is not possible using other thermostable polymerases due to their propensity for non-templated incorporation. Thus, these enzymes are suitable for use in automated amplification systems such as PCR.

10272981 10272982 10272983 10272984 10272985

Sources of DNA

Suitable sources of DNA, including a variety of cells, tissues, organs or organisms, may be obtained through any number of commercial sources (including American Type Culture Collection (ATCC), Rockville, Maryland; Jackson Laboratories, Bar Harbor, Maine; Cell Systems, Inc., Kirkland, Washington; Advanced Tissue Sciences, La Jolla, California). Cells that may be used as starting materials for genomic DNA preparation are preferably eukaryotic (including fungi or yeasts, plants, protozoans and other parasites, and animals including humans and other mammals). Although any mammalian cell may be used for preparation of DNA, preferred are blood cells (erythrocytes and leukocytes), endothelial cells, epithelial cells, neuronal cells (from the central or peripheral nervous systems), muscle cells (including myocytes and myoblasts from skeletal, smooth or cardiac muscle), connective tissue cells (including fibroblasts, adipocytes, chondrocytes, chondroblasts, osteocytes and osteoblasts) and other stromal cells (e.g., macrophages, dendritic cells, Schwann cells), although other cells, including the progenitors, precursors and stem cells that give rise to the above-described somatic cells, are equally suitable. Also suitable for use in the preparation of DNA are mammalian tissues or organs such as those derived from brain, kidney, liver, pancreas, blood, bone marrow, muscle, nervous, skin, genitourinary, circulatory, lymphoid, gastrointestinal and connective tissue sources, as well as those derived from a mammalian (including human) embryo or fetus. These cells, tissues and organs may be normal, or they may be pathological such as those involved in infectious diseases (caused by bacteria, fungi or yeast, viruses (including AIDS) or parasites), in genetic or biochemical pathologies (e.g., cystic fibrosis, hemophilia, Alzheimer's disease, schizophrenia, muscular dystrophy or multiple sclerosis), or in cancerous processes.

More specifically, in one aspect of the invention, the relationship between a first individual and a second individual may be determined by analyzing and typing a particular polymorphic DNA fragment, such as a minisatellite or microsatellite DNA sequence. In such a method, the amplified fragments for each

100-900-800-700-600-500-400-300-200-100

5
10
15
20
25
30

individual are compared to determine similarities or dissimilarities. Such an analysis is accomplished, for example, by comparing the size of the amplified fragments from each individual, or by comparing the sequence of the amplified fragments from each individual. In another aspect of the invention, genetic identity can be determined. Such identity testing is important, for example, in paternity testing, forensic analysis, etc. In this aspect of the invention, a sample containing DNA (e.g., a crime scene sample or a sample from an individual) is analyzed and compared to a sample from one or more individuals. In one such aspect of the invention, one sample of DNA may be derived from a first individual and another sample may be derived from a second individual whose relationship to the first individual is unknown; comparison of these samples from the first and second individuals by the methods of the invention may then facilitate a determination of the genetic identity or relationship between the first and second individual. In a particularly preferred such aspect, the first DNA sample may be a known sample derived from a known individual and the second DNA sample may be an unknown sample derived, for example, from crime scene material. In an additional aspect of the invention, one sample of DNA may be derived from a first individual and another sample may be derived from a second individual who is related to the first individual; comparison of these samples from the first and second individuals by the methods of the invention may then facilitate a determination of the genetic kinship of the first and second individuals by allowing examination of the Mendelian inheritance, for example, of a polymorphic, minisatellite, microsatellite or STR DNA fragment. In another aspect of the invention, DNA fragments important as genetic markers for encoding a gene of interest can be identified and isolated. For example, by comparing samples from different sources, DNA fragments which may be important in causing diseases such as infectious diseases (of bacterial, fungal, parasitic or viral etiology), cancers or genetic diseases, can be identified and characterized. In this aspect of the invention a DNA sample from normal cells or tissue is compared to a DNA sample from diseased cells or tissue. Upon comparison according to the invention, one

TO2250-0021650

or more unique polymorphic fragments present in one DNA sample and not present in the other DNA sample can be identified and isolated. Identification of such unique polymorphic fragments allows for identification of sequences associated with, or involved in, causing the diseased state.

5 Once the starting cells, tissues, organs or other samples are obtained, DNA may be prepared therefrom by methods that are well-known in the art (See, e.g., Maniatis, T., et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, pp. 9.16-9.23 (1989); Kaufman, P.B., et al., *Handbook of Molecular and Cellular Methods in Biology and Medicine*, Boca Raton, Florida: CRC Press, pp. 1-26 (1995)). The DNA samples thus prepared may then be used to identify, analyze and type polymorphic DNA fragments, including minisatellite, microsatellite and STR DNA fragments, by amplification, preferably by PCR amplification, as modified by the methods of the present invention.

10 General methods for amplification and analysis of DNA fragments are well-known to one of ordinary skill in the art (see, e.g., U.S. Pat. Nos. 4,683,195; 4,683,202; and 4,800,159; Innis, M.A., et al., eds., *PCR Protocols: A Guide to Methods and Applications*, San Diego, California: Academic Press, Inc. (1990); Griffin, H.G., and Griffin, A.M., eds., *PCR Technology: Current Innovations*, 15 Boca Raton, Florida: CRC Press (1994)). Typically, these methods comprise contacting the DNA sample with a thermostable DNA polymerase in the presence of one or more primer sequences, amplifying the DNA sample to generate a collection of amplified polymorphic, minisatellite, microsatellite or STR DNA fragments, preferably by PCR or equivalent automated amplification technique, 20 separating the amplified DNA fragments by size, preferably by gel electrophoresis, and analyzing the gels for the presence of polymorphic, minisatellite, microsatellite or STR DNA fragments by direct comparison of the pattern of fragments generated from a first sample of DNA to those from a second sample of DNA, or by a more indirect comparison using known size markers.

TO2/250-33376362

As noted above, amplification protocols used heretofore for analyzing and typing polymorphic DNA fragments, particularly minisatellite, microsatellite or STR DNA sequences, use certain thermostable DNA polymerases such as *Taq* (U.S. Patent Nos. 4,683,195; 4,683,202; and 4,800,159). However, as discussed in detail above, these approaches yield amplification products in which one or more non-templated nucleotides is added to the 3' termini of the products by the polymerases, thus leading to heterogeneity in the amplification products, and ambiguity concerning the correct size of the amplification products.

This problem is overcome in the present invention by contacting the DNA sample in the amplification reaction mixtures with one or more DNA polymerases of the invention which are reduced, substantially reduced or eliminated in the ability to add a nontemplated 3' terminal nucleotide to the growing strand. Preferably, such DNA polymerases are *Thermotoga* DNA polymerases, more preferably a *Thermotoga* DNA polymerase substantially reduced in 3'-5' exonuclease activity, still more preferably a *Tne* polymerase (SEQ ID NOs:1,2), a *Tma* polymerase (U.S. Patent No. 5,374,553), or a mutant or derivative thereof, and most preferably one of the following mutants of *Tne* polymerase: *Tne* N'Δ219, D323A (SEQ ID NO:3); *Tne* N'Δ283, D323A (SEQ ID NO:4); *Tne* N'Δ192, D323A (SEQ ID NO:5); *Tne* D137A, D323A (SEQ ID NO:6); *Tne* D8A, D323A (SEQ ID NO:7); *Tne* G195D, D323A (SEQ ID NO:8); *Tne* G37D, D323A (SEQ ID NO:9); *Tne* N'Δ283 (SEQ ID NO:10); *Tne* D137A, D323A, R722K; *Tne* D137A, D323A, R722Y; *Tne* D137A, D323A, R722L; *Tne* D137A, D323A, R722H; *Tne* D137A, D323A, R722Q; *Tne* D137A, D323A, F730Y; *Tne* D137A, D323A, K726R; *Tne* D137A, D323A, K726H; *Tne* D137A, D323A, R722K, F730Y; *Tne* D137A, D323A, R722K, K726R; *Tne* D137A, D323A, R722K, K726H; *Tne* D137A, D323A, R722H, F730Y; *Tne* D137A, D323A, R722H, K726R; *Tne* D137A, D323A, R722Q, F730Y; *Tne* D137A, D323A, R722Q, K726R; *Tne* D137A, D323A, R722Q, K726H; *Tne* D137A, D323A, R722N, F730Y; *Tne* D137A, D323A, R722N, K726R; *Tne* D137A, D323A, R722N, K726H; *Tne* D137A,

TO/290-32236860

5

10

15

20

25

30

D323A, F730S; *The N'Δ283*, D323A, R722K/H/Q/N/Y/L; *The N'Δ219*, D323A, R722K; *The N'Δ219*, D323A, F730Y; *The N'Δ219*, D323A, K726R; *The N'Δ219*, D323A, K726H; *The D137A*, D323A, F730S, R722K/Y/Q/N/H/L, K726R/H; *The D137A*, D323A, F730T, R722K/Y/Q/N/H/L, K726R/H; *The D137A*, D323A, F730T; *The F730S*; *The F730A*; *The K726R*; *The K726H*; and *The D137A*, D323A, R722N.

It will be understood, however, that other thermostable DNA polymerases or mutants thereof, any of which are reduced, substantially reduced, or eliminated in the ability to add a non-templated 3' terminal nucleotide to the growing strand, may be used in the methods of the present invention equivalently. The DNA polymerases are used in the methods of the present invention at a concentration of about 0.0001 units/ml to about 10 units/ml, preferably at a concentration of about 0.001 units/ml to about 5 units/ml, more preferably at a concentration of about 0.004 units/ml to about 1 unit/ml, and most preferably at a concentration of about 0.04 units/ml. Thus, the methods of the present invention produce a population of amplified DNA fragments, most preferably of polymorphic or microsatellite DNA fragments, which comprise substantially no non-templated 3' terminal nucleotides. By "substantially no non-templated 3' terminal nucleotides" is meant that the population of amplified DNA fragments demonstrates about 0-50%, about 0-30%, about 0-20%, preferably about 0-10%, more preferably about 0-5%, still more preferably about 0-1% and most preferably about 0%, of DNA molecules containing non-templated 3' nucleotides compared to amplified DNA fragments produced by the polymerase control. When testing the ability of a DNA polymerase to add 3' non-templated nucleotides, the polymerase, when it has substantially reduced or eliminated 3' exonuclease activity, is compared to *Taq* DNA polymerase (see above). When testing polymerases which have been modified or mutated to reduce or eliminate 3' non-templated nucleotide addition, the mutated or modified polymerase is compared to the corresponding wildtype, unmodified or unmutated polymerase (see above).

Following amplification by the methods of the present invention, the amplified DNA fragments may be analyzed to identify or type a polymorphic, minisatellite, microsatellite or STR DNA fragment. This step is usually accomplished by separation of the amplified DNA fragments by size, a procedure which permits the determination of the presence of unique polymorphic fragments in one or more of the DNA samples. The fragments may be separated by any physical or biochemical means including gel electrophoresis, capillary electrophoresis, chromatography (including sizing, affinity and immunochromatography), density gradient centrifugation and immunoabsorption. For carrying out the present invention, separation of DNA fragments by gel electrophoresis is particularly preferred, as it provides a rapid and highly reproducible means of sensitive separation of a multitude of DNA fragments, and permits direct, simultaneous comparison of the fragments in several samples of DNA, or samples of DNA from a first and a second individual.

Gel electrophoresis is typically performed on agarose or polyacrylamide sequencing gels according to standard protocols, preferably using gels containing polyacrylamide at concentrations of 3-12% and most preferably at about 8%, and containing urea at a concentration of about 4-12M, most preferably about 8M. Samples are loaded onto the gels, usually with samples containing amplified DNA fragments prepared from different sources of genomic DNA being loaded into adjacent lanes of the gel to facilitate subsequent comparison. Reference markers of known sizes may be used to facilitate the comparison of samples. Following electrophoretic separation, DNA fragments may be visualized and identified by a variety of techniques that are routine to those of ordinary skill in the art, such as autoradiography. One can then examine the autoradiographic films either for differences in polymorphic fragment patterns ("typing") or for the presence of one or more unique bands in one lane of the gel ("identifying"); the presence of a band in one lane (corresponding to a single sample, cell or tissue type) that is not observed in other lanes indicates that the DNA fragment comprising that unique band is source-specific and thus a potential polymorphic DNA fragment.

卷之三

5 A variety of DNA fragments comprising polymorphic, minisatellite, microsatellite or STR DNA fragments can thus be identified using the methods of the present invention by comparing the pattern of bands on the films depicting various samples. Importantly, using the present methods the amplification

10 products of the polymorphic DNA fragments will be faithful copies of the template (allele) material -- *i.e.*, they will not exhibit undesired additional nucleotides at their 3' termini via non-templated addition of nucleotides by the polymerases. One can extend this approach, in another preferred embodiment, to isolate and characterize these fragments or any DNA fragment amplified without the non-templated addition of a 3' terminal nucleotide. In this embodiment, one or more of the unique DNA fragments are removed from the gel which was used for identification (see above), according to standard techniques such as electroelution or physical excision.

15 The isolated unique DNA fragments may then be inserted into standard nucleotide vectors, including expression vectors, suitable for transfection or transformation of a variety of prokaryotic (bacterial) or eukaryotic (yeast, plant or animal including human and other mammalian) cells. In particular, the present invention provides methods of cloning such isolated unique DNA fragments, or any PCR-amplified DNA fragment, by blunt-end cloning. As described above,

20 *Taq* DNA polymerase adds a non-templated nucleotide, typically a deoxyadenosine ("A"), to the 3' terminus of the amplified DNA fragment. Thus, *Taq*-catalyzed PCR generates a collection of DNA fragments with 3' A overhangs. To clone such *Taq*-amplified fragments, two approaches are commonly used: either the 3' A overhang is removed by treating the amplified fragment with, for example, T4 DNA polymerase (a technique sometimes called "3' polishing"), or a special cloning vector with a 3' T overhang (a "TA cloning vector") is used. Of course, such approaches are more time-consuming and expensive than if direct insertion of the amplified fragment were done. Such a direct approach is possible using the methods of the invention, which generates little or no 3' A overhangs

25 (and thus, blunt ends) on the amplified DNA fragments. The DNA fragments,

102290-32268800

amplified according to the methods of the invention, may thus be directly inserted into corresponding blunt-ended vectors according to standard techniques (for example, using T4 DNA ligase). Thus, the present invention provides a method of blunt-end cloning of a DNA fragment that obviates the use of TA cloning vectors or 3' polishing.

To identify the presence of minisatellite DNA fragments, the polymorphic DNA fragments that are identified and isolated by the methods of the present invention may be further characterized, for example by sequencing (*i.e.*, determining the nucleotide sequence of the polymorphic fragments), by methods described above and others that are standard in the art (*see, e.g.*, U.S. Patent Nos. 4,962,022 and 5,498,523, which are directed to methods of DNA sequencing).

Kits

The invention also provides kits for use in the identification, analysis and typing of a polymorphic DNA fragment, particularly a minisatellite or STR DNA fragment, according to the present methods. Kits according to the present invention may comprise a carrying means being compartmentalized to receive in close confinement therein one or more containers such as vials, tubes, bottles and the like. Each of such containers may comprise components or a mixture of components needed to perform DNA amplification or analysis.

Such kits may comprise of one or more thermostable DNA polymerases reduced, substantially reduced or eliminated in the ability to add a non-templated 3' nucleotide to a growing DNA strand. Preferably the container contains a *Thermotoga* DNA polymerase or a mutant or a derivative thereof, particularly those described in full detail above. The kit may also contain one or more DNA primer molecules, one or more deoxyribonucleoside triphosphates needed to synthesize a DNA molecule complementary to a DNA template, and/or a buffer suitable for amplification of a nucleic acid molecule (or combinations thereof).

A kit for DNA analysis may include one or more of the above components, and may further include containers which contain reagents necessary for

TO/2001-00076860

separation and analysis of DNA fragments, such as polyacrylamide, agarose, urea, detergents and the like.

5 Of course, it is also possible to combine one or more of these reagents in a single tube. A detailed description of such formulations at working concentrations is described in co-pending U.S. Application No. 08/689,815 of Ayoub Rashtchian and Joseph Solus, entitled "Stable Compositions for Nucleic Acid Amplification and Sequencing" filed on August 14, 1996, the disclosure of which is incorporated by reference herein in its entirety.

10 The invention also relates to kits for detectably labeling molecules, sequencing, amplifying and synthesizing molecules by well known techniques. See U.S. Patent Nos. 4,962,020, 5,173,411, 4,795,699, 5,498,523, 5,405,776 and 5,244,797. Such kits may comprise a carrying means being compartmentalized to receive in close confinement one or more container means such as vials, test tubes and the like. Each of such container means comprises components or a mixture of components needed to perform nucleic acid synthesis, sequencing, labeling, or amplification.

15 A kit for sequencing DNA may comprise a number of container means. Such a kit may comprise one or more of the polymerases of the invention, one or a number of types of nucleotides needed to synthesize a DNA molecule complementary to DNA template, one or a number of different types of terminators (such as dideoxynucleoside triphosphates), a pyrophosphatase, one or a number of primers and/or a suitable sequencing buffer (or combinations of such components).

20 A kit used for amplifying or synthesizing of nucleic acids will comprise, one or more polymerases of the invention, and one or a number of nucleotides or mixtures of nucleotides. Various primers may be included in a kit as well as a suitable amplification or synthesis buffers.

25 When desired, the kit of the present invention may also include container means which comprise detectably labeled nucleotides which may be used during the synthesis or sequencing of a nucleic acid molecule. One of a number of labels

T012301-325CT6860

10

15

20

25

30

may be used to detect such nucleotides. Illustrative labels include, but are not limited to, radioactive isotopes, fluorescent labels, chemiluminescent labels, bioluminescent labels and enzyme labels.

Use of the Methods and Kits

The polymerases, methods and kits embodied in the present invention will have general utility in any application utilizing nucleic acid amplification methodologies, particularly those directed to the analysis and typing of polymorphic or minisatellite DNA fragments, and most particularly those directed to the analysis and typing of minisatellite, microsatellite and STR DNA fragments. Amplification techniques in which the present methods may be used include PCR (U.S. Patent Nos. 4,683,195 and 4,683,202), Strand Displacement Amplification (SDA; U.S. Patent No. 5,455,166; EP 0 684 315), and Nucleic Acid Sequence-Based Amplification (NASBA; U.S. Patent No. 5,409,818; EP 0 329 822). Nucleic acid analysis and typing techniques which may employ the present compositions include nucleic acid sequencing methods such as those disclosed in U.S. Patent Nos. 4,962,022 and 5,498,523, as well as more complex PCR-based nucleic acid fingerprinting techniques such as Random Amplified Polymorphic DNA (RAPD) analysis (Williams, J.G.K., *et al.*, *Nucl. Acids Res.* 18(22):6531-6535, 1990), Arbitrarily Primed PCR (AP-PCR; Welsh, J., and McClelland, M., *Nucl. Acids Res.* 18(24):7213-7218, 1990), DNA Amplification Fingerprinting (DAF; Caetano-Anollés *et al.*, *Bio/Technology* 9:553-557, 1991), and microsatellite PCR or Directed Amplification of Minisatellite-region DNA (DAMD; Heath, D.D., *et al.*, *Nucl. Acids Res.* 21(24): 5782-5785, 1993). In particular, the polymerases, methods and kits of the present invention will be useful in the fields of medical genetics, therapeutics and diagnostics, forensics (particularly identity and paternity testing), and agricultural (e.g., plant breeding) and other biological sciences, in any procedure utilizing DNA polymerases for analysis and typing of polymorphic, minisatellite, microsatellite or STR DNA fragments. Particularly suitable for diagnosis by the methods of the present

102/390-33376860

invention are genetic diseases such as cystic fibrosis, hemophilia, Alzheimer's disease, schizophrenia, muscular dystrophy or multiple sclerosis. Together, these abilities will assist medical professionals and patients in diagnostic and prognostic determinations as well as in the development of treatment and prevention regimens for these and other disorders.

It will also be apparent to one of ordinary skill in the art that the present methods may be used to screen animal tissues to be subsequently used in medical procedures such as tissue or organ transplants, blood transfusions, zygote implantations and artificial inseminations. In such procedures, pre-screening of the subject tissues for the presence of particular polymorphic DNA fragments may improve the success of tissue or organ transplants (by decreasing the likelihood of rejection due to donor-recipient genetic incompatibility) and of zygote implantations (by eliminating the use of genetically defective zygotes). Similarly, use of these methods will reduce the chances of transmission of infectious diseases (e.g., hepatitis and AIDS) in medical procedures that are often prone to such transmission, such as blood transfusions and artificial insemination. Finally, use of the present invention for identification of unique polymorphic, minisatellite, microsatellite and STR DNA fragments will assist in forensic science in such applications as crime-scene analysis of blood, tissue and body secretions containing small amounts of DNA, as well as in paternity testing.

It will be readily apparent to those skilled in the relevant arts that other suitable modifications and adaptations to the methods and applications described herein are obvious and may be made without departing from the scope of the invention or any embodiment thereof. Having now described the present invention in detail, the same will be more clearly understood by reference to the following examples, which are included herewith for purposes of illustration only and are not intended to be limiting of the invention.

07/02/2004 222516860

Example 1: Bacterial Strains And Growth Conditions

Thermotoga neapolitana DSM No. 5068 was grown under anaerobic conditions as described in the DSM catalog (addition of resazurin, Na_2S , and sulfur granules while sparging the media with nitrogen) at 85°C in an oil bath from 12 to 24 hours. The cells were harvested by filtering the broth through Whatman #1 filter paper. The supernatant was collected in an ice bath and then centrifuged in a refrigerated centrifuge at 8,000 rpm for twenty minutes. The cell paste was stored at -70°C prior to total genomic DNA isolation.

E. coli strains were grown in 2X LB broth base (Lennox L broth base: GIBCO/BRL) medium. Transformed cells were incubated in SOC (2% tryptone, 0.5% yeast extract, yeast 10 mM NaCl , 2.5 mM KCl , 20mM glucose, 10mM MgCl_2 , and 10mM MgSO_4 per liter) before plating. When appropriate antibiotic supplements were 20 mg/l tetracycline and 100 mg/l ampicillin. *E. coli* strain DH10B (Lorow *et al.*, *Focus* 12:19-20 (1990)) was used as host strain. Competent DH10B may be obtained from Life Technologies, Inc. (LTI) (Rockville, MD).

Example 2: DNA Isolation

Thermotoga neapolitana chromosomal DNA was isolated from 1.1g of cells by suspending the cells in 2.5 ml TNE (50mM Tris-HCl, pH 8.0, 50mM NaCl , 10mM EDTA) and treated with 1% SDS for 10 minutes at 37°C. DNA was extracted with phenol by gently rocking the lysed cells overnight at 4°C. The next day, the lysed cells were extracted with chloroform:isoamyl alcohol. The resulting chromosomal DNA was further purified by centrifugation in a CsCl density gradient. Chromosomal DNA isolated from the density gradient was extracted three times with isopropanol and dialyzed overnight against a buffer containing 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (TE).

10/29/01 ECCR6960

Example 3: Construction of Genomic Libraries

The chromosomal DNA isolated in Example 2 was used to construct a genomic library in the plasmid pCP13. Briefly, 10 tubes each containing 10 μ g of *Thermotoga neapolitana* chromosomal DNA was digested with 0.01 to 10 units of *Sau*IIIAl for 1 hour at 37°C. A portion of the digested DNA was tested in an agarose (1.2%) gel to determine the extent of digestion. Samples with less than 50% digestion were pooled, ethanol precipitated and dissolved in TE. 6.5 μ g of partially digested chromosomal DNA was ligated into 1.5 μ g of pCP13 cosmid which had been digested with *Bam*HI restriction endonuclease and dephosphorylated with calf intestinal alkaline phosphatase. Ligation of the partially digested *Thermotoga* DNA and *Bam*HI cleaved pCP13 was carried out with T4 DNA ligase at 22°C for 16 hours. After ligation, about 1 μ g of ligated DNA was packaged using λ -packaging extract (obtained from Life Technologies, Inc., Rockville, MD). DH10B cells (Life Tech. Inc.) were then infected with 100 μ l of the packaged material. The infected cells were plated on tetracycline containing plates. Serial dilutions were made so that approximately 200 to 300 tetracycline resistant colonies were obtained per plate.

*Example 4: Screening for Clones Expressing *Thermotoga neapolitana* DNA Polymerase*

Identification of the *Thermotoga neapolitana* DNA polymerase gene of the invention was cloned using the method of Sagner *et al.*, *Gene* 97:119-123 (1991) which reference is herein incorporated in its entirety. Briefly, the *E. coli* tetracycline resistant colonies from Example 3 were transferred to nitrocellulose membranes and allowed to grow for 12 hours. The cells were then lysed with the fumes of chloroform:toluene (1:1) for 20 minutes and dried for 10 minutes at room temperature. The membranes were then treated at 95°C for 5 minutes to inactivate the endogenous *E. coli* enzymes. Surviving DNA polymerase activity

TO/290-25276860

was detected by submerging the membranes in 15 ml of polymerase reaction mix (50 mM Tris-HCl (pH 8.8), 1 mM MgCl₂, 3 mM β -mercaptoethanol, 10 μ M dCTP, dGTP, dTTP, and 15 μ Ci of 3,000 Ci/mmol [α^{32} P]dATP) for 30 minutes at 65°C.

Using autoradiography, three colonies were identified that expressed a *Thermotoga neapolitana* DNA polymerase. The cells were grown in liquid culture and the protein extract was made by sonication. The presence of the cloned thermostable polymerase was confirmed by treatment at 90°C followed by measurement of DNA polymerase activity at 72°C by incorporation of radioactive deoxyribonucleoside triphosphates into acid insoluble DNA. One of the clones, expressing *Tne* DNA polymerase, contained a plasmid designated pCP13-32 and was used for further study.

Example 5: Subcloning of *Tne* DNA polymerase

Since the pCP13-32 clone expressing the *Tne* DNA polymerase gene contains about 25 kb of *T. neapolitana* DNA, subcloning a smaller fragment of the *Tne* polymerase gene was attempted. The molecular weight of the *Tne* DNA polymerase purified from *E. coli*/pCP13-32 was about 100 kd. Therefore, a 2.5-3.0 kb DNA fragment will be sufficient to code for full-length polymerase. A second round of *Sau*3A partial digestion similar to Example 3 was done using pCP13-32 DNA. In this case, a 3.5 kb region was cut out from the agarose gel, purified by Gene Clean (BIO 101, La Jolla, CA) and ligated into plasmid pSport 1 (Life Technologies, Inc.) which had been linearized with *Bam*HI and dephosphorylated with calf intestinal alkaline phosphatase. After ligation, DH10B was transformed and colonies were tested for DNA polymerase activity as described in Example 1. Several clones were identified that expressed *Tne* DNA polymerase. One of the clones (pSport-*Tne*) containing about 3 kb insert was further characterized. A restriction map of the DNA fragment is shown in Fig. 1. Further, a 2.7 Kb *Hind*III-*Sst*I fragment was subcloned into pUC19 to generate

pUC19-*Tne*. *E. coli*/pUC19-*Tne* also produced *Tne* DNA polymerase. *E. coli* DH10B (pUC19-*Tne*) was deposited on September 30, 1994 with the Collection, Agricultural Research Culture Collection (NRRL), 1815 Peoria, IL 61604 as Deposit No. NRRL B-21338. The nucleotide and amino acid sequence of *Tne* polymerase is described in U.S. application serial nos. 08/706,702 and 08/706,706 filed September 9, 1996, both of which are incorporated by reference herein.

5

Example 6: Purification of *Thermotoga neapolitana* DNA Polymerase from *E. coli*

10

Twelve grams of *E. coli* cells expressing cloned *Tne* DNA polymerase (DH10B/pSport-*Tne*) were lysed by sonication (four thirty-second bursts with a medium tip at the setting of nine with a Heat Systems Ultrasonics Inc., model 375 sonicator) in 20 ml of ice cold extraction buffer (50 mM Tris HCl (pH 7.4), 8% glycerol, 5 mM mercaptoethanol, 10 mM NaCl, 1 mM EDTA, 0.5 mM PMSF). The sonicated extract was heated at 80°C for 15 min. and then cooled in ice for 5 min. 50 mM KCl and PEI (0.4%) was added to remove nucleic acids. The extract was centrifuged for clarification. Ammonium sulfate was added to 60%, the pellet was collected by centrifugation and resuspended in 10 ml of column buffer (25 mM Tris-HCl (pH 7.4), 8% glycerol, 0.5% EDTA, 5 mM 2-mercaptoethanol, 10 mM KCl). A Blue-Sepharose (Pharmacia) column, or preferably a Toso heparin (Tosohaas) column, was washed with 7 column volumes of column buffer and eluted with a 15 column volume gradient of buffer from 10mM to 2 M KCl. Fractions containing polymerase activity were pooled. The fractions were dialyzed against 20 volumes of column buffer. The pooled fractions were applied to a Toso650Q column (Tosohaas). The column was washed to baseline OD₂₈₀ and elution effected with a linear 10 column volume gradient of 25 mM Tris (pH 7.4), 8% glycerol, 0.5 mM EDTA, 10 mM KCl, 5 mM β-mercaptoethanol to the same buffer plus 650 mM KCl. Active fractions were pooled.

15

20

25

TO/290-2506860

Example 7: Construction of *Thermotoga neapolitana* 3'-to-5' Exonuclease Mutant

The amino acid sequence of portions of the *The* DNA polymerase was compared with other known DNA polymerases such as *E. coli* DNA polymerase 1, *Taq* DNA polymerase, T5 DNA polymerase, and T7 DNA polymerase to localize the regions of 3'-to-5' exonuclease activity, and the dNTP binding domains within the DNA polymerase. One of the 3'-to-5' exonuclease domains was determined based on the comparison of the amino acid sequences of various DNA polymerases (Blanco, L., et al. *Gene* 112: 139-144 (1992); Braithwaite and Ito, *Nucleic Acids Res.* 21: 787-802 (1993)) is as follows:

<i>The</i>	318	PSFALDLETSS	328	(SEQ ID NO:18)
Pol I	350	PVFAFDTETDS	360	(SEQ ID NO:19)
T5	159	GPVAFDSETSA	169	(SEQ ID NO:20)
T7	1	MIVSDIEANA	10	(SEQ ID NO:21)

As a first step to make the *The* DNA polymerase devoid of 3'-to-5' exonuclease activity, a 2kb *Sph* fragment from pSport-*The* was cloned into M13mp19 (LTI, Rockville, MD). The recombinant clone was selected in *E. coli* DH5 α FIQ (LTI, Rockville, MD). One of the clones with the proper insert was used to isolate uracilated single-stranded DNA by infecting *E. coli* CJ236 (Biorad, California) with the phage particle obtained from *E. coli* DH5 α FIQ. An oligonucleotide, GA CGT TTC AAG CGC TAG GGC AAA AGA (SEQ ID NO:22) was used to perform site directed mutagenesis. This site-directed mutagenesis converted Asp³²³ (indicated as * above) to Ala³²³. An Eco47III restriction site was created as part of this mutagenesis to facilitate screening of the mutant following mutagenesis. The mutagenesis was performed using a protocol as described in the Biorad manual (1987) except T7 DNA polymerase was used instead of T4 DNA polymerase (USB, Cleveland, OH). The mutant clones were

102/290-223148860

screened for the *Eco*47III restriction site that was created in the mutagenic oligonucleotide. One of the mutants having the created *Eco*47III restriction site was used for further study. The mutation Asp³²³ to Ala³²³ was confirmed by DNA sequencing.

5 To incorporate the 3'→5' exonuclease mutation in an expression vector, the mutant phage was digested with *Sph*I and *Hind*III. A 2 kb fragment containing the mutation was isolated. This fragment was cloned in pUC-Tne to replace the wild type fragment. See Figure 2A. The desired clone, pUC-Tne (3'→5'), was isolated. The presence of the mutant sequence was confirmed by the presence of the unique *Eco*47III site. The plasmid was then digested with *Sst*I and *Hind*III. The entire mutant polymerase gene (2.6 kb) was purified and cloned into *Sst*I and *Hind*III digested pTrc99 expression vector (Pharmacia, Sweden). The clones were selected in DH10B (LTI, Rockville, MD). The resulting plasmid was designated pTrcTne35. See Figure 2B. This clone produced active heat stable DNA polymerase.

10

15

Example 8: Phenylalanine to Tyrosine Mutant

20 The polymerase active site including the dNTP binding domain is usually present at the carboxyl terminal region of the polymerase. The sequence of the *Tne* polymerase gene suggests that the amino acids that presumably contact and interact with the dNTPs are present within the 694 bases starting at the internal *Bam*HI site. See Figure 1. This conclusion is based on homology with a prototype polymerase *E. coli* DNA polymerase I. See Poliskiy et al., *J. Biol. Chem.* 265:14579-14591 (1990). A comparison was made of the O-helix for various polymerases:

DRAFT - 06/27/00

	<i>The</i>	722	RRVGKVMVNFSIIYG	735	(SEQ ID NO:12)
	Pol I	754	RRSAKAINFGLIYG	767	(SEQ ID NO:13)
	T5	562	RQAAKAITFGILYG	575	(SEQ ID NO:14)
	T7	518	RDNAKTFIYGFLYG	531	(SEQ ID NO:15)
5	<i>Tag</i>	659	RRAAKTINFGVLYG	672	(SEQ ID NO:16)

10 In order to change Phe⁷³⁰ of the *The* polymerase to a Tyr⁷³⁰ site directed mutagenesis was performed using the oligonucleotide GTA TAT TAT AGA GTA GTT AAC CAT CTT TCC A (SEQ ID NO:23). As part of this oligonucleotide directed mutagenesis, a *Hpa*I restriction site was created in order to screen mutants easily. The same uracilated single-stranded DNA and mutagenesis procedure described in Example 7 were used for this mutagenesis. Following mutagenesis, the mutants were screened for the *Hpa*I site. Mutants with the desired *Hpa*I site were used for further study. The mutation was confirmed by DNA sequencing.

15 The Phe⁷³⁰ to Tyr⁷³⁰ mutation was incorporated into pUC-*The* by replacing the wild type *Sph*I -*Hind*III fragment with the mutant fragment obtained from the mutant phage DNA. The presence of the desired clone, pUC-TneFY, was confirmed by the presence of the unique *Hpa*I site, see Figure 2A. The entire mutant polymerase gene was subcloned into pTrc99 as an *Sst*I-*Hind*III fragment 20 as described above in DH10B. The resulting plasmid was designated pTrcTneFY. (Figure 2B). The clone produced active heat stable polymerase.

Example 9: 3'→5' Exonuclease and Phe⁷³⁰→Tyr⁷³⁰ Double Mutants

25 In order to introduce the 3'→5' exonuclease mutation and the Phe⁷³⁰→Tyr⁷³⁰ mutation in the same expression vector, pTrc99, it was necessary to first reconstitute both mutations in the pUC-Tne clone. See Figure 3. Both the pUC-Tne (3'→5') and the pUC-TneFY were digested with *Bam*HI. The digested pUC-Tne (3'→5') was dephosphorylated to avoid recirculation in the following

102390123376360

ligations. The resulting fragments were purified on a 1% agarose gel. The largest *Bam*HI fragment (4.4 kb) was purified from pUC-Tne (3'→5') digested DNA and the smallest *Bam*HI fragment (0.8 kb) containing the Phe⁷³⁰→Tyr⁷³⁰ mutation was purified and ligated to generate pUC-Tne35FY. The proper orientation and the presence of both mutations in the same plasmid was confirmed by *Eco*47III, *Hpa*I, and *Sph*I-*Hind*III restriction digests. See Figure 3.

The entire polymerase containing both mutations was subcloned as a *Sst*I-*Hind*III fragment in pTrc99 to generate pTrcTne35FY in DH10B. The clone produced active heat stable polymerase.

10 **Example 10: 3'→5' Exonuclease, 5'→3' Exonuclease, and Phe⁷³⁰→Tyr⁷³⁰ Triple Mutants**

In most of the known polymerases, the 5'-to-3' exonuclease activity is present at the amino terminal region of the polymerase (Ollis, D.L., *et al.*, *Nature* 313, 762-766, 1985; Freemont, P.S., *et al.*, *Proteins* 1, 66-73, 1986; Joyce, C.M., *Curr. Opin. Struct. Biol. 1*: 123-129 (1991). There are some conserved amino acids that are implicated to be responsible for 5'-to-3' exonuclease activity (Gutman and Minton, *Nucl. Acids Res.* 21, 4406-4407, 1993). *See supra*. It is known that 5'-to-3' exonuclease domain is dispensable. The best known example is the Klenow fragment of *E. coli* Pol I. The Klenow fragment is a natural proteolytic fragment devoid of 5'-to-3' exonuclease activity (Joyce, C.M., *et al.*, *J. Biol. Chem.* 257, 1958-1964, 1990). In order to generate an equivalent mutant for *Tne* DNA polymerase devoid of 5'-to-3' exonuclease activity, the presence of a unique *Sph*I site present 680 bases from the *Sst*I site was exploited. pUC-Tne35FY was digested with *Hind*III, filled-in with Klenow fragment to generate a blunt-end, and digested with *Sph*I. The 1.9 kb fragment was cloned into an expression vector pTTQ19 (Stark, M.J.R., *Gene* 51, 255-267, 1987) at the *Sph*I-*Sma*I sites and was introduced into DH10B. This cloning strategy generated an in-frame polymerase clone with an initiation codon for methionine from the

TO2202132076860

vector. The resulting clone is devoid of 219 amino terminal amino acids of *Tne* DNA polymerase. This clone is designated as pTTQTne535FY (Fig. 4). The clone produced active heat stable polymerase. No exonuclease activity could be detected in the mutant polymerase as evidenced by lack of presence of unusual sequence ladders in the sequencing reaction. This particular mutant polymerase is highly suitable for DNA sequencing.

5
10
15
20
25

Example 11: 5'->3' Exonuclease Deletion and Phe⁷³⁰→Tyr⁷³⁰ Substitution Mutant

In order to generate the 5'-to-3' exonuclease deletion mutant of the *Tne* DNA polymerase Phe⁷³⁰→Tyr⁷³⁰ mutant, the 1.8 kb *Sph*I-*Spe*I fragment of pTTQTne535FY was replaced with the identical fragment of pUC-Tne FY. See Fig. 4. A resulting clone, pTTQTne5FY, produced active heat stable DNA polymerase. As measured by the rate of degradation of a labeled primer, this mutant has a modulated, low but detectable, 5'-to-3' exonuclease activity compared to wild type *Tne* DNA polymerase. M13/pUC Forward 23-Base Sequencing PrimerTM, obtainable from LTI, Rockville, MD, was labeled at the 5' end with [P^{32}] ATP and T4 kinase, also obtainable from LTI, Rockville, MD, as described by the manufacturer. The reaction mixtures contained 20 units of either wildtype or mutant *Tne* DNA polymerase, 0.25 pmol of labeled primer, 20 mM tricine, pH 8.7, 85 mM potassium acetate, 1.2 mM magnesium acetate, and 8% glycerol. Incubation was carried out at 70°C. At various time points, 10 μ l aliquots were removed to 5 μ l cycle sequencing stop solution and were resolved in a 6 % polyacrylamide sequencing gel followed by andoradiography. While the wildtype polymerase degraded the primer in 5 to 15 minutes, it took the mutant polymerase more than 60 minutes for the same amount of degradation of the primer.

TO/290-2331880

Example 12: Purification of the Mutant Polymerases

The purification of the mutant polymerases was done essentially as described Example 6, *supra*, with minor modifications. Specifically, 5 to 10 grams of cells expressing cloned mutant *Tne* DNA polymerase were lysed by sonication with a Heat Systems Ultrasonic, Inc. Model 375 machine in a sonication buffer comprising 50 mM Tris-HCl (pH 7.4); 8% glycerol; 5 mM 2-mercaptoethanol, 10 mM NaCl, 1 mM EDTA, and 0.5 mM PMSF. The sonication sample was heated at 75°C for 15 minutes. Following heat treatment, 200 mM NaCl and 0.4% PEI was added to remove nucleic acids. The extract was centrifuged for clarification.

Ammonium sulfate was added to 48%, the pellet was resuspended in a column buffer consisting of 25 mM Tris-HCl (pH 7.4); 8% glycerol; 0.5% EDTA; 5 mM 2-mercaptoethanol; 10 mM KCl and loaded on a heparin agarose (LTI) column. The column was washed with 10 column volumes using the loading buffer and eluted with a 10 column volume buffer gradient from 10 mM to 1 M KCl. Fractions containing polymerase activity were pooled and dialyzed in column buffer as above with the pH adjusted to 7.8. The dialyzed pool of fractions were loaded onto a MonoQ (Pharmacia) column. The column was washed and eluted as described above for the heparin column. The active fractions are pooled and a unit assay was performed.

The unit assay reaction mixture contained 25 mM TAPS (pH 9.3), 2 mM MgCl₂, 50 mM KCl, 1 mM DTT, 0.2 mM dNTPs, 500 µg/ml DNase I treated salmon sperm DNA, 21 mCi/ml [α P³²] dCTP and various amounts of polymerase in a final volume of 50 µl. After 10 minutes incubation at 70°C, 10 µl of 0.5 M EDTA was added to the tube. TCA perceptible counts were measured in GF/C filters using 40 µl of the reaction mixture.

Example 13: Generation of 5'-to-3' exonuclease mutant of full length *Tne* DNA polymerase

1. Identification of Two Amino Acids Responsible for 5'-to-3' Exonuclease Activity

Tne DNA polymerase contains three enzymatic activities similar to *E. coli* DNA polymerase I: 5'-to-3' DNA polymerase activity, 3'-to-5' exonuclease activity and 5'-to-3' exonuclease activity. This example is directed to the elimination of the 5'-to-3' exonuclease activity in full length *Tne* DNA polymerase. Gutman and Minton (*Nucleic Acids Res.* 1993, 21, 4406-4407) identified six (A-F) conserved 5'-to-3' exonuclease domains containing a total of 10 carboxylates in various DNA polymerases in the polI family. Seven out of 10 carboxylates (in domains A, D and E) have been implicated to be involved in divalent metal ions binding as judged from the crystal structure (Kim et al. *Nature*, 1995, 376, 612-616) of *Taq* DNA polymerase. However, there was no clear demonstration that these carboxylates are actually involved 5'-to-3' exonuclease activity. In order to find out the biochemical characteristics of some of these carboxylates, two of the aspartic acids in domains A and E were chosen for mutagenesis. The following aspartic acids in these two domains were identified:

Tne DNA polymerase: 5 FLFD⁸GT 10 (domain A) (SEQ ID NO:24)

Taq DNA polymerase: 15 LLVD¹⁸GH 20 (SEQ ID NO:25)

and

Tne DNA polymerase: 132 SLITGD¹³⁷KDML141 (domain E) (SEQ ID NO:26)

Taq DNA polymerase: 137 RILTAD¹⁴²KDLY146 (SEQ ID NO:27)

25 2. Isolation of Single Stranded DNA for Mutagenesis

Single stranded DNA was isolated from pSportTne (see *infra*). pSportTne was introduced into DH5 α F'IQ (LTI, Rockville, MD) by transformation. A single

1027293-29316660

colony was grown in 2 ml Circle Grow (Bio 101, CA) medium with ampicillin at 37°C for 16 hrs. A 10 ml fresh media was inoculated with 0.1 ml of the culture and grown at 37°C until the A590 reached approximately 0.5. At that time, 0.1 ml of M13KO7 helper phage (1X10¹¹ pfu/ml, LTI) was added to the culture. The infected culture was grown for 75 min. Kanamycin was then added at 50 µg/ml, and the culture was grown overnight (16 hrs.). The culture was spun down. 9 ml of the supernatant was treated with 50 µg each of RNaseA and DNaseI in the presence of 10 mM MgCl₂ for 30 min. at room temperature. To this mixture, 0.25 volume of a cocktail of 3M ammonium acetate plus 20% polyethylene glycol was added and incubated for 20 min. on ice to precipitate phage. The phage was recovered by centrifugation. The phage pellet was dissolved in 200 µl of TE (10 mM Tris-HCl (pH 8) and 1 mM EDTA). The phage solution was extracted twice with equal volume of buffer saturated phenol (LTI, Rockville, MD), twice with equal volume of phenol:chloroform:isoamyl alcohol mixture (25:24:1, LTI, Rockville, MD) and finally, twice with chloroform: isoamyl alcohol (24:1). To the aqueous layer, 0.1 volume of 7.5 M ammonium acetate and 2.5 volume of ethanol were added and incubated for 15 min. at room temperature to precipitate single stranded DNA. The DNA was recovered by centrifugation and suspended in 200 µl TE.

20 3. **Mutagenesis of D⁸ and D¹³⁷**

Two oligos were designed to mutagenize D⁸ and D¹³⁷ to alanine. The oligos are: 5' GTAGGCCAGGGCTGT**GCCGG**CAAAGAGAAATAGTC 3' (D8A) (SEQ ID NO:28) and 5' GAAGCATATCCTT**GGCGCCGG**TAT TATGAAAATC 3' (D137A) (SEQ ID NO:29). In the D8A oligo a *Ngo*AIV (bold underlined) and in the oligo D137A a *Kas*I (bold underlined) site was created for easy identification of clones following mutagenesis. 200 pmol of each oligo was kinased according to the Muta-gene protocol (Bio-Rad, CA) using 5 units of T4 Kinase (LTI, Rockville, MD). 200 ng of single stranded DNA was

10/26/2000

annealed with 2 pmol of oligo according to the Muta-gene protocol. The reaction volume was 10 μ l. Following the annealing step, complementary DNA synthesis and ligation was carried out using 5 units of wildtype T7 DNA polymerase (USB, Ohio) and 0.5 unit T4 ligase (LTI). 1 μ l of the reaction was used to transform a MutS *E. coli* (obtainable from Dr. Paul Modrich at the Duke University, NC) and selected in agar plates containing ampicillin. A control annealing and synthesis reaction was carried out without addition of any oligo to determine the background. There were 50-60 fold more colonies in the transformation plates with the oligos than without any oligo. Six colonies from each mutagenic oligo directed synthesis were grown and checked for respective restriction site (*NgoAIV* or *KasI*). For D8A (*NgoAIV*), 4 out of 6 generated two fragments (3 kb and 4.1 kb). Since pSportTne has an *NgoAIV* site near the f1 intergenic region, the new *NgoAIV* site within the *Tne* DNA polymerase produced the expected fragments. The plasmid was designated as pSportTne*NgoAIV*. For D137A (*KasI*), 5 out of 6 clones produced two expected fragments of 1.1 kb and 6 kb in size. Since pSportTne has another *KasI* site, the newly created *KasI* site generated these two expected fragments. The plasmid was designated as pSportTne*KasI*. Both D8A and D137A mutations were confirmed by DNA sequencing.

4. Reconstruction of the Mutant Polymerase into Expression Vector

During the course of expression of *Tne* DNA polymerase or mutant *Tne* DNA polymerase, a variety of clones were constructed. One such clone was designated as pTTQ *Tne* SeqS1. This plasmid was constructed as follows: first, similar to above mutagenesis technique glycine 195 was changed to an aspartic acid in pSportTne. A mutation in the corresponding amino acid in *E. coli* DNA polymeraseI (polA214, domain F) was found to have lost the 5'-to-3' exonuclease activity (Gutman and Minton, see above). An *Ssp*I site was created in the mutant polymerase. Second, a 650 bp *Ssp*I-*Sph*I fragment containing the G195D mutation was subcloned in pUCTne35FY (see *infra*) to replace the wild type fragment.

This plasmid was called pUCTne3022. Finally, the entire mutant *Tne* DNA polymerase was subcloned from pUCTne3022 into pTTQ18 as *Sst*I-*Hind*III fragment to generate pTTQTneSeqS1. To introduce the mutation D8A or D137A in this expression vector, the 650 bp *Sst*I-*Sph*I was replaced with the same *Sst*I-*Sph*I fragment from pSportTneNgoAIV or pSportTneKasI. The plasmids were designated as pTTQTneNgo(D8A) and pTTQTneKas(D137A), respectively.

5 **5. Confirmation of the Mutations by DNA Sequencing**

DNA sequencing of both mutant polymerases confirmed the presence of the restriction site *Ngo*AIV as well as the mutation D8A; and *Kas*I site as well as the mutation D137A. Also confirmed by DNA sequencing was the presence of the mutation D323A and the *Eco*47III restriction site in the 3'-to-5' exonuclease region. In addition, confirmed by DNA sequencing was the F730Y mutation and the *Hpa*I restriction site in the O-helix region of the mutant *Tne* DNA polymerase.

10 **6. 5'-to-3' exonuclease Activity of the Mutant *Tne* DNA Polymerases**

15 The full length mutant DNA polymerase was purified as described above. The 5'-to-3' exonuclease activity was determined as described in the LTI catalog. Briefly, 1 pmol of labeled (32 P) *Hae*III digested λ DNA (LTI) was used for the assay. The buffer composition is: 25 mM Tris-HCl (pH 8.3), 5 mM MgCl₂, 50 mM NaCl, 0.01% gelatin. The reaction was initiated by the addition of 0, 2, 4, 6 and 10 units of either wild type or mutant *Tne* DNA polymerase in a 50 μ l reaction. The reaction mix was incubated for 1 hr at 72°C. A 10 μ l aliquot was subjected to PEI-cellulose thin layer chromatography and the label released was quantitated by liquid scintillation. In this assay, both D8A and D137A mutants showed less than 0.01% label release compared to the wild type *Tne* DNA polymerase. The result demonstrates that in both D8A and D137A mutants the 5'-to-3' exonuclease activity has been considerably diminished. Thus, it has been

TO/290-38876960

10

15

20

25

confirmed that these two aspartates are involved with the 5'-to-3' exonuclease activity.

Example 14: Generation of double mutants, R722K/F730Y, R722Q/F730Y, R722H/F730Y and R722N/F730Y of Tne DNA polymerase

For all mutations, the PCR method was used. A common 5'-oligo, CAC CAG ACG ***GGT ACC*** GCC ACT GGC AGG TTG (SEQ ID NO:30), was used. This oligo contains a *Kpn*I site (shown above in bold italics). The template used for PCR was pTTQTneSeqS1 (Example 13) which already contains the F730Y mutation in the Tne polymerase gene. For the R722K/F730Y mutation, the oligo used was TAT AGA GTA ***GTT AAC*** CAT CTT TCC AAC CCG TTT CAT TTC TTC GAA CAC (SEQ ID NO:31). For the R722Q/F730Y mutation, the oligo used was TAT AGA GTA ***GTT AAC*** CAT CTT TCC AAC CCG TTG CAT TTC TTC GAA CAC (SEQ ID NO:32). For the R722N/F730Y mutation, the oligo used was TAT AGA GTA ***GTT AAC*** CAT CTT TCC AAC CCG GTT CAT TTC TTC GAA CAC (SEQ ID NO:33) and for the R722H/F730Y the oligo used was TAT AGA GTA ***GTT AAC*** CAT CTT TCC AAC CCG ATG CAT TTC TTC GAA CAC (SEQ ID NO:34). Each of these oligos contains a *Hpa*I site (bold italics). The underlined codons were the mutated codons for arginine at the position 722 for respective amino acids. The PCR generated a 318 bp product containing a *Kpn*I and a *Hpa*I site. The PCR products were digested with *Kpn*I and *Hpa*I and cloned into pUC-TneFY digested with *Kpn*I and *Hpa*I to replace the original fragment to generate pUC19TneFY-R722K, pUC19TneFY-R722Q, pUC19TneFY-R722H and pUC19TneFY-R722N. Finally, the *Kpn*I-HindIII fragment (~800bp) of pTTQTneKasI(D137A) was replaced by the ~800 bp *Kpn*I-HindIII fragment from these plasmids to generate pTne11 (R722K/F730Y), pTne10 (R722Q/F730Y), pTne13 (R722H/F730Y) and pTne9 (R722N/F739Y), respectively. The mutations were confirmed by DNA sequencing.

TO/290-35376860

Example 15: Generation of *Tne* DNA Polymerase mutants *F730A* and *F730S*

F730A was constructed using PCR. The forward oligo was AAG ATG **GTT AAC GCG TCT ATA ATA TAC GG** (SEQ ID NO:35) which contains a *Hpa*I site and a *Mlu*I site (bold italics). The reverse oligo was CAA GAG GCA CAG AGA GTT TCA CC (SEQ ID NO:36) which anneals downstream of *Spe*I present in the *Tne* polymerase gene. The template used for PCR was pTTQTne KasI (D137A). The 482bp PCR product was digested with *Hpa*I and *Spe*I and cloned into pUC-TneFY thereby replacing the amino acid tyrosine at position 730 with alanine. This construct was called pUC-Tne FA.

F730S was constructed by site directed mutagenesis. The oligo was GTA TAT TAT AGA GGA **GTT AAC CAT CTT TCC** (SEQ ID NO:37) where a *Hpa*I site was created (bold italics). The single stranded DNA used was isolated from pSport-Tne that contains the double mutation D137A and D323A. This construct was designated pTne 47. The *Tne* polymerase gene was then cloned as an *Ss*I and *Hind*III fragment into the plasmid pUC19 and the resulting clone was designated pTne101.

Example 16: Generation of *Tne* DNA polymerase with a *Hpa*I site in front of the amino acid phenylalanine at position 730.

A construct of *Tne* polymerase was made using PCR where a *Hpa*I restriction enzyme site was introduced into the gene in front of the amino acid phenylalanine at position 730. The forward oligonucleotide was AAG ATG **GTT AAC TTC TCT ATA ATA TAC GG** (SEQ ID NO:38) which contains a *Hpa*I site (shown above in bold italics) and the reverse oligo was the same as in Example 15 above. The template used for PCR was pTne33 which contains the *Tne* polymerase gene with D137A and D323A mutations cloned in pUC19. The 482bp PCR product was digested with *Hpa*I and *Spe*I and was used to replace the corresponding fragment in pTne101 (see example 15). The construct was

TOZ28032216260

sequenced to verify that the amino acid at position 730 was indeed phenylalanine and the plasmid was numbered pTne106.

Example 17: Generation of double mutants R722Y/F730A and R722L/F730A of the Tne DNA polymerase.

For both the mutations PCR method was used. The common 5' oligo was the same as in Example 14. For R722Y/F730A mutation the oligo used was TAT AGA GTA **GTT AAC** CAT CTT TCC AAC CCG GTA **CAT GTC** TTC GTT CAC (SEQ ID NO:39). For R722L/F730A mutation the oligo used was TAT AGA GTA **GTT AAC** CAT CTT TCC AAC CCG CAA **CAT GT C** TTC GTT CAC (SEQ ID NO:40). Each of these oligos contain a *Hpa*I site (shown above in bold italics). The underlined codons were the mutated codons for arginine at the position 722 for respective amino acids. An *Afl*III site was also created (shown above in bold italics next to the underlined codon) in order to confirm the mutation. The PCR generated a 318 bp product containing a *Kpn*I and a *Hpa*I site. The PCR products were digested with *Kpn*I and *Hpa*I and cloned into pUC-TneFA (see example 15). The constructs were named as pUCTneYA and pUCTneLA.

Example 18: Generation of Tne DNA Polymerase mutants R722Y and R722L.

The plasmid pTne 106 (see example 16) was digested with *Hpa*I and *Kpn*I and the 318 bp fragment was replaced with the corresponding fragment from pUCTneYA or pUCTneLA (see Example 17) to generate the mutants R722Y or R722L. In these constructs the amino acid at position 730 is the same as wild type *Tne* (phenylalanine). The constructs were sequenced to confirm the R722Y and the R722L mutations. The *Tne* DNA polymerase gene was then cloned as a *Sst*I/*Hind*III fragment into the plasmid pSport1.

Example 19: Generation of The DNA Polymerase mutants R722K, R722Q and R722H.

The construct pTne 106 (see example 16) was digested with *Hpa*I and *Kpn*I and the 318 bp fragment was replaced with the corresponding fragment from the construct pUC19TneFY-R722K, pUC19TneFY-R722H or pTne10 (see Example 14), to generate the mutants R722K, R722H and R722Q. The constructs were sequenced to confirm the mutations. The *The* DNA polymerase gene was then subcloned into the vector pSport1 as a *Sst*I/*Hind*III fragment.

Example 20: Purification of the mutant *Tne* DNA Polymerases

The purification of the mutants of *The* DNA polymerase was carried out based on the method described above with minor modifications. Two to three grams of cells expressing cloned mutant *The* DNA polymerase were resuspended in 15-20 ml of sonication buffer (50 mM Tris-HCl, pH 8.0, 10% glycerol, 5mM 2-mercaptoethanol, 50 mM NaCl, 1 mM EDTA, and 0.5 mM PMSF and sonicated with a 550 Sonic Dismembrator (Fisher Scientific). The sonicated sample was heated at 82°C for 20 min and then cooled in ice-water for 5 min. In the sample, 20 mM NaCl and 0.2% PEI were added and centrifuged at 13,000 rpm for 10 min. Ammonium sulfate (305g/L) was added to the supernatant. The pellet was collected by centrifugation and resuspended in 4 ml of MonoQ column buffer (50 mM Tris-HCl, pH 8.0, 10% glycerol, 5mM 2-mercaptoethanol, 50 mM NaCl, and 1 mM EDTA). The sample was dialyzed against one liter of MonoQ buffer overnight. Following the centrifugation at 13,000 rpm to remove any insoluble materials, the sample was loaded onto a MonoQ column (HR5/5, Pharmacia). The column was washed with MonoQ column buffer to baseline of OD₂₈₀ and then eluted with a linear gradient of 50-300 mM NaCl in 20 ml MonoQ column buffer. The fractions were analyzed by 8% SDS-PAGE and the *The* DNA

Y0728012535198000

polymerase activity determined as described earlier. The fractions containing active and pure *Taq* DNA polymerase were pooled.

Example 21: Generation of *Taq* DNA Polymerase Mutants R659K, R659H and R659Y

5 A 2.5 kb portion of the gene encoding *Taq* DNA polymerase (Figure 5) was cloned as a *Hind* III-*Xba* I fragment into M13mp19. Site directed mutagenesis was performed using the BioRad mutagene kit (BioRad California) using the following oligonucleotides:

10 CTTGGCCGCCCG***AT***GCATCAGGGGGTC (SEQ ID NO:41) for the R659H mutation where an *Nsi*I site was created (see bold italics);

CTTGGCCGCCCG***C***TT***CAT***GAGGGGGTCCAC (SEQ ID NO:42) for the R659K mutation where a *Bsp*HI site was created (see bold italics); and

CTTGGCCGCC***CTGTAC***ATCAGGGGGTC (SEQ ID NO:43) for the R659Y mutation where a *Bsr*GI site was created (see bold italics).

15 For each mutation, six clones were screened by analyzing the M13RF DNA for the expected restriction sites. Mutations were confirmed by DNA sequencing. DNA shown to contain the mutation by the presence of the expected restriction site was digested with *Ngo*AIV and *Xba* I and the approximately 1600 base pair fragment was used to replace corresponding fragment in the wildtype 20 *Taq* DNA polymerase gene. These constructs were made in a plasmid containing *Taq* polymerase gene under the control of Tac promoter (pTTQ *Taq*) to generate pTTQ *Taq* (R659K), pTTQ *Taq* (R659H) and pTTQ *Taq* (R659Y). These plasmids were transformed into *E. coli* DH10B (LTI).

10/22/2012 10:22:50 AM

Example 22: Construction of Tne polymerase mutants containing F730S and F730T

Single stranded DNA was isolated from pSportTne (Tne35) containing D137A and D323A mutations as described in the section 2 of example 13. These D137 and D323A mutations rendered Tne DNA polymerase devoid of 5'-exonuclease and 3'-to-5'-exonuclease activities, respectively. Thus, Tne 35 is devoid of both exonuclease activities. The site-directed mutagenesis was done following the protocol decribed in section 3 of Example 13. The oligos used were 5' GTA TAT TAT AGA GGA **GTT AAC** CAT CTT TCC 3' (SEQ ID NO:37) for F730S and 5' GTA TAT TAT AGA GGT **GTT AAC** CAT CTT TCC 3' (SEQ ID NO:44) for F730T. Each of these two oligos contain a diagnostis *Hpa*I site for screening of mutants in the MutS strain. The mutant plasmids were transferred to DH10B strains. The mutations were finally confirmed by DNA sequencing. The mutant polymerases were purified by the procedure as described in Example 20.

Example 23: Determination of the Activity of Non-templated One Base Addition for Tne and Tag DNA Polymerase by Primer Extension Assay

The following 34-mer primer was ^{32}P labeled at the 5' end with [γ - ^{32}P] ATP and T4 polynucleotide kinase by standard protocol (Molecular Cloning, A Laboratory Manual, Cold Spring Harbor, NY):

5'-GGGAGACCGGAATTCTCCTTCATTAATTCCTATA-3' (SEQ ID NO:45)

The unincorporated ATP was removed by a BioRad P6 column(1.0 ml). The labeled primer was annealed to the following homogenous (purified) 48-mer template:

5'-TGGAGACCCTGGAACTATAGGAATTAAATGAAGGAGAATTCCGGT
CTCCC-3' (SEQ ID NO:46).

Wildtype or mutant DNA polymerases (0.125-1.0 unit) were incubated at 72°C for 2 min in 20 mM Tris-HCl (pH8.3), 1.5 mM MgCl₂, 50 mM KCl, 1.0 mM DTT, 200 uM of dCTP, dGTP, TTP, dATP, and 0.02 pmol of the annealed primer-template. After addition of sequencing stop buffer and heated at 90°C for 2 min, the mixture was loaded onto 10% polyacrylamide-7 M urea. Following the electrophoresis, the gel was dried and the reaction products were analyzed by autoradiography. The non-templated one base addition products shown in Figure 6 were quantified by a PhosphorImager (Molecular Dynamics).

Tne DNA polymerases % of N+1

1	D137A	18.5
2	D137A D323A	78.5
3	D137A D323A R722K	0.7
4	D137A D323A R722Y	0.7
5	D137A D323A R722L	5.7
6	D137A D323A R722H	1.2
7	D137A D323A R722Q	1.4
8	D137A D323A F730Y	61.3
9	D137A D323A R722 K F730Y	6.8
10	D137A D323A R722H F730Y	2.1
11	D137A D323A R722Q F730Y	6.1
12	D137A D323A R722N F730Y	15.9
13	D137A D323A F730S	8.3
14	D137A D323A F730T	24.2

Taq DNA Polymerases		% of N+1
1	W.T.	37
2	R659K	1.4
3	R659Y	0.9
5	4 R659H	0.5
	5 F667Y	39.1

Example 24: Comparison of DNA Synthesis by *Taq* and *Tne*

To examine its propensity to add a nontemplated nucleotides to the 3' termini of PCR products, *Tne* DNA polymerase (5'exo⁻, 3'exo⁻) was compared side-by-side with *Taq* DNA polymerase in amplifications of short tandem repeats at 23 different marker loci (see Table 1). Reactions comprising 20 mM TRIS-HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, 200 mM each dNTP, 200 nM [³²P] α -dATP, 200 nM each of the upper and lower primers, 25 ng of human DNA, 0.1% nonionic detergent and 1 unit of DNA polymerase (in a volume of 25 μ l) were assembled on ice. Published sequences for upper and lower primers for each locus, as shown in Table 1, were used for all amplifications.

Reactions were loaded into a Perkin Elmer model 9600 thermocycler preheated to 94°C and PCR was done using standard cycling conditions (1 minute pre-denaturation at 94°C; 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 1 minute at 72°C; 1 minute post-extension at 72°C; overnight soak at 4°C). A portion of each reaction was mixed with an equal volume of 95% formamide containing dyes to indicate the progress of electrophoresis. Samples were heated to 90°C for 2 min, and 5 μ l of each was loaded on a 6% denaturing polyacrylamide gel. Sequencing ladders were loaded to provide size markers, and electrophoresis was performed at 70 watts. After electrophoresis the gel was transferred to filter paper and dried. Autoradiography and phosphoimage analysis was performed to visualize the PCR products and estimate the percentage of

TOZ290-22375860

product which contained the added nucleotide by direct comparison of bands produced by each enzyme.

Examples of the side-by-side comparisons of amplification products produced by *Taq* DNA polymerase and *The* DNA polymerase are shown in Figure 7 for the CD4 locus and in Figure 8 for the D20S27 locus. At both of these loci, a significant portion of the *Taq* PCR product contained an extra non-templated nucleotide (n+1), while *The* polymerase demonstrated no apparent non-templated nucleotide incorporation for either the CD4 locus (Figure 7) or the D20S27 locus (Figure 8). Complete results for the 23 marker loci examined are summarized in Table 2. In PCRs using *Taq* DNA polymerase, a portion of the amplification product contained an extra non-templated nucleotide (n+1) at every locus examined. In PCRs using *The* DNA polymerase, however, no detectable portion of the product at any of the loci examined contained an additional non-templated nucleotide. These results indicate that *The* DNA polymerase, in contrast to *Taq* DNA polymerase, is substantially reduced in the ability to add a non-templated 3' terminal nucleotide to the growing strand. Since the *The* DNA polymerase used in these amplifications was a 3'exo- mutant (*i.e.*, it was substantially reduced in 3' exonuclease activity), these results are consistent with the notion that the *The* polymerase was unable to add the extra nucleotide to the product rather than adding the nucleotide and then removing it via a 3' exonuclease activity.

09891232-052701

5

10

15

20

Table 1. Primers Used in Example 24.

Locus	Upper Primer (SEQ ID No.)	Lower Primer (SEQ ID No.)	Reference
D13S71	GTATTTGGTATGGTGTGTC (47)	CTATTTGGAAATAATGGCT (48)	Nucl. Acids Res. 18:4638 (1990)
D13S13	ACGAACTTACAGTAC (49)	TITCAGAGAACTAACCTGT (50)	Nucl. Acids Res. 18:2199 (1990)
D15S87	GATAATGCCAAAATGTTG (51)	TGCTCAGGATTCTCTCA (52)	Nucl. Acids Res. 18:4640 (1990)
D21S16	AGCTTGAGACCTCTGTGTC (53)	ATTCAGAAACAGTGTGT (54)	Nature Genet. 7:246-339 (1994)
CD4	TGAGTGTGCAAGCTGAACTAGC (55)	GCTGAGTGTGAGAGTGAAGAC (56)	Nucl. Acids Res. 19:4791 (1991)
FLA2A	CCACTAGTGTGAAAGTCCATGA (57)	TACATGTGCCAGGCTGTCTCA (58)	Nucl. Acids Res. 18:7468 (1990)
D10S49	ACTCATAAAGTGACAGTC (59)	GTTGTTGACTATGAT (60)	Nucl. Acids Res. 18:1927 (1990)
D4S175	ATCTCTGTCCTCCCTGT (61)	CITATTCGCTTGAAGTAG (62)	Genomics 14:205-219 (1992)
AP0C2AC	AGCCCGTGTGAGACATGACT (63)	TACATAGGAGAACCTCATCTCCC (64)	Hum. Genet. 83:245-251 (1989)
D20S27	TTTATGGAGCTATGGATA (65)	CACCCATTGATCTTGAAAG (66)	Nucl. Acids Res. 18:2202 (1990)
D15S127	CCAAACCAACTGGAA (67)	AAACAGTTGCCAACCGTT (68)	Nature Genet. 7:246-339 (1994)
D15S398	CATCAAATGCTGACTGGTA (69)	TCAATTATGTCAGGCCAAAT (70)	Nature Genet. 7:246-339 (1994)
AP0C2	CATAGCGAGACTCATCTCC (71)	GGGAGAGGGCAAAAGTCAT (72)	Am. J. Hum. Genet. 44:388-396 (1989)
D10S89	AACACTAGTGTACATTTCA (73)	AGCTTGGCCTGAGGCTCT (74)	Nucl. Acids Res. 18:4637 (1990)
VWA	CCCTAGTGGATGATAAGATAATC (75)	GGACAGATGATAATACATGGATGG (76)	Hum. Molec. Genet. 1:287 (1992)
D16S01	TTCTCTTACAAACTGGCCC (77)	ATTGGGATGGCTGAGAG (78)	Nature Genet. 7:246-339 (1994)
D7S440	ACATCTTAAGACTTCCAAAT (79)	AGAGCATGACCCCTGAATG (80)	Nucl. Acids Res. 18:4039 (1990)
D4S174	AAGAAACCACTGCAATGACT (81)	CATTCCTTAAATGGTAAAGC (82)	Nucl. Acids Res. 18:4636 (1990)
D16S520	GCTTAACTACAGGGGG (83)	TCCAACGCCATGTAAACC (84)	Nature Genet. 7:246-339 (1994)
D16S511	CCCGGGAGCAAGTCA (85)	CAGCCAAAAGCAGATA (86)	Nature Genet. 7:246-339 (1994)
D21S11	ATATGGTGTCACTCCCAAG (87)	TGTTATGTCATGTTCTCCAG (88)	Hum. Molec. Genet. 1:57 (1992)
TH01	CAGCTGCCCTAATGCAAC (89)	GCTCCGAGTGGAGGTCA (90)	Nucl. Acids Res. 19:3753 (1991)
ACTBP2	ATTCCTGGCCACAAAGTGA (91)	ACATCTCCCTACCCCTATA (92)	Nucl. Acids Res. 20:1432 (1992)

Table 2. Non-templated 3' Terminal Nucleotide Addition by *Taq* and *Tne* DNA Polymerases at 23 Microsatellite DNA Loci.

Locus	Repeat Type	<i>Taq</i> (% n+1)	<i>Tne</i> (% n+1)
D13S71	dinucleotide	100	0
D18S103	dinucleotide	75-100	0
D15S87	dinucleotide	30-50	0
D2S136	dinucleotide	90-100	0
HUMCD4	pentanucleotide	50	0
HUMPLA2A	trinucleotide	25	0
D19S49	dinucleotide	75	0
D4S175	dinucleotide	75	0
APOC2AC	dinucleotide	50	0
D20S27	dinucleotide	50	0
D15S127	dinucleotide	100	0
D4S398	dinucleotide	50	0
APOC2	dinucleotide	50	0
D10S89	dinucleotide	75-100	0
HUMVWA	tetranucleotide	90	0
D16S401	dinucleotide	100	0
D7S440	dinucleotide	90	0
D4S174	dinucleotide	75	0
D16S520	dinucleotide	100	0
D16S511	dinucleotide	100	0
HUMD21S11	tetranucleotide	100	0
HUMTHO1	tetranucleotide	75	0
HUMACTBP2	tetranucleotide	25	0

TO/290-2251036

5
10
15
20
25

Example 25: Comparison of DNA Synthesis by *Tne* and Other Thermostable Enzymes

To further evaluate the differences in the propensities of *Tne* and other thermostable DNA polymerases to add non-templated 3' terminal nucleotides to PCR products, side-by-side amplifications were performed using a single marker locus DIS103 and a variety of thermostable enzymes, including 3' exonuclease deficient (3'exo-) enzymes, and 3' exonuclease competent (3'exo+) enzymes. PCR amplifications, electrophoresis and analysis were performed as described for Example 24, using 200 nM of D1S103-specific upper and lower primers.

Results for the amplifications using 3'exo- DNA polymerases are shown in Table 3. With the exception of *Tne*(3'exo-), all of the 3'exo- DNA polymerases examined exhibited a propensity to add a non-templated 3' terminal nucleotide (n+1) to the PCR product. For *Taq* and *Tbr* DNA polymerases, up to 100% of the PCR products contained an additional non-templated 3' terminal nucleotide, while *Vent*, Deep *Vent*, and *Dtok* 3'exo- mutants polymerases added this non-templated nucleotide to 25-100% of the PCR products. In contrast, the 3'exo-mutant of *Tne* DNA polymerase was substantially reduced in the ability to add a nontemplated 3' terminal nucleotide to the DNA molecule; none of the PCR products from reactions using *Tne*(3'exo-) had an additional non-templated nucleotide at their 3' termini.

Results from amplifications using 3'exo+ DNA polymerases are shown in Table 4. Five polymerases were examined as well as two commercially available enzyme mixes (mixtures of a primary 3'exo- polymerase and a secondary 3'exo+ polymerase). At this locus, the 3'exo+ DNA polymerases (*Tne*, *Tma*, *Pfu*, *Pwo* and 9°North) yielded product which did not contain an extra non-templated nucleotide. The enzyme mixtures (Elongase and Expand HiFi) yielded a mixture of products with and without an additional non-templated nucleotide. Together, these results indicate that *Tne* polymerases, whether 3'exo- or 3'exo+, are substantially reduced in the ability to add a nontemplated 3' terminal nucleotide to the DNA molecule. Moreover, of the preferred 3'exo- polymerases, only *Tne*(3'exo-) was substantially reduced in this activity, indicating its favorableness

10 09894332862781

5

15

20

25

30

in PCR applications where non-templated nucleotide addition to the amplification product is undesirable.

Table 3. Non-templated 3' Terminal Nucleotide Addition by 3'exo-DNA Polymerases.

Enzyme	n Sized Fragment	n+1 Sized Fragment
<i>Tne</i> (3'exo-)	+	-
<i>Taq</i>	-	+
<i>Vent</i> (3'exo-)	+	+
Deep <i>Vent</i> (3'exo-)	+	+
<i>Dtol</i> (3'exo-)	+	+
<i>Thermolase</i> <i>Tbr</i>	-	+

Table 4. Non-templated 3' Terminal Nucleotide Addition by 3'exo+DNA Polymerases.

Enzyme	n Sized Fragment	n+1 Sized Fragment
<i>Tne</i> (3'exo+)	+	-
<i>Ultma</i>	+	-
<i>Pfu</i>	+	-
<i>Pwo</i>	+	-
9°North	+	-
Elongase	+	+
Expand HiFi	+	+

Example 26: Comparison of DNA Synthesis by *Tne* Mutants

To examine the utility of *Tne* DNA polymerase and various mutants thereof in amplification of microsatellite DNA sequences, the experiments described in Example 25 were repeated with 11 different *Tne* DNA polymerase

mutants. Of these mutants, 3 were 5'exo+, while the remainder were 5'exo- either due to N-terminal deletions of the protein, or to point mutations in the 5' exonuclease domain of the polymerase.

As shown in Table 5, use of the 5'exo- *Tne* mutants resulted in productive amplifications, yielding PCR products with no non-templated 3' terminal nucleotide additions. Results were identical for all seven *Tne*(3'exo-/5'exo-) polymerase mutants, as well as for the single *Tne*(3'exo+/5'exo-) mutant tested. Results with 5'exo+ *Tne* mutants were inconclusive under the conditions tested.

These results indicate that the mutants of *Tne* DNA polymerase tested in the present studies are substantially reduced in the ability to add nontemplated 3' terminal nucleotides to the growing strand, particularly a DNA template comprising a microsatellite DNA sequence or an STR.

Table 5. Non-templated 3' Terminal Nucleotide Addition by Tne DNA Polymerase Mutants

Enzyme	5'exo Activity	3'exo Activity	n Sized Fragment	n+1 Sized Fragment
Tne N'Δ219, D323A	-	-	+	-
Tne N'Δ283, D323A	-	-	+	-
Tne N'Δ192, D323A	-	-	+	-
Tne D137A, D323A	-	-	+	-
Tne D8A, D323A	-	-	+	-
Tne G195D, D323A	-	-	+	-
Tne G37D, D323A	-	-	+	-
Tne N'Δ283	-	+	+	-

Example 27: Fluorescent Analysis of DNA Synthesis by *Tne* and *Taq* DNA Polymerases

In an alternative analysis approach, the propensities of *Taq* DNA polymerase and *Tne* DNA polymerase to add non-templated nucleotides to the PCR products were compared using fluorescent detection. The polymerases were compared in side-by-side amplifications utilizing a commonly used commercially available marker panel (ABI Prism Linkage Mapping Set Panel 21), examining ten different loci. Reaction mixtures (15 ml) containing 1.5 mM MgCl₂, 250 mM of each deoxynucleoside triphosphate, 333 nM of each primer, 50 ng of human DNA and 0.6 units of *Taq* or *Tne* DNA polymerase were assembled on ice. Reactions were loaded into a Perkin Elmer model 9600 thermocycler preheated to 95°C, and PCR was performed using recommended cycling conditions (5 minutes pre-denaturation at 95°C; 10 cycles of 15 seconds at 95°C, 15 seconds at 55°C, and 60 seconds at 72°C; and 20 cycles of 15 seconds at 89°C, 15 seconds at 55°C, and 60 seconds at 72°C). Two sets of extension reactions were conducted for each locus, one with a 10 minute post-extension incubation at 72°C followed by an overnight soak and storage at 4°C (conditions which favor nontemplated 3' nucleotide addition), the other with no post-extension incubation followed by immediate storage at -20°C (conditions which inhibit nontemplated 3' nucleotide addition). A portion of each reaction was diluted, mixed with loading cocktail, heat denatured and loaded on an 8% polyacrylamide sequencing gel. The ABI 373 Stretch Automated Sequencer was run for 5-6 hours at 15W in order to obtain single base resolution, and data were analyzed using GeneScan software. Areas of the peaks recognized by the software were used to estimate the percentage of nontemplated 3' nucleotide addition ("n+1") for each locus by the two polymerases under the two different extension conditions. The total area under the allelic peaks was used to compare the yields of specific PCR product obtained in *Tne* and *Taq* amplifications, and yields produced by *Tne* polymerase were expressed for each locus as a percentage of those produced by *Taq* polymerase. Table 6 summarizes the results obtained.

TOZ290=ZCCT5860

Table 6. Comparison of DNA Amplification by *Taq* and *Tne* DNA Polymerases by Fluorescent Detection

	locus	color	expected size	cycling conditions	<i>Taq</i> pattern	<i>Tne</i> pattern	<i>Tne</i> yield (% <i>Taq</i>)
5	D16S40 5	blue	107-145	no final ext 10' final ext	0% n+1 94% n+1	100% n 100% n	89% 178%
	D16S12 7	green	114-148	no final ext 10' final ext	53% n+1 100% n+1	100% n 100% n	133% 142%
	D16S52 0	yellow	144-160	no final ext 10' final ext	40% n+1 100% n+1	98% n 100% n	275% 252%
10	D16S51 1	green	182-222	no final ext 10' final ext	62% n+1 100% n+1	100% n 100% n	51% 160%
	D16S41 1	blue	215-235	no final ext 10' final ext 10' final ext	0% n+1 64% n+1 67% n+1	100% n 100% n 95% n	218% 257% 305%
15	D16S13 1	yellow	237-275	no final ext 10' final ext	0% n+1 69% n+1	100% n 95% n	48% 231%
	D16S13 0	blue	280-294	no final ext	0% n+1	100% n	101%
20	D16S50 3	yellow	294-310	no final ext 10' final ext	0% n+1 73% n+1	100% n 100% n	102% 166%
	D16S11 7	green	316-334	no final ext 10' final ext	17% n+1 77% n+1	100% n 100% n	130% 326%
	D16S51 5	blue	320-350	no final ext 10' final ext	32% n+1 100% n+1	100% n 100% n	486% 298%

1027290-2002000000000000

The results shown in Table 6 confirm that under conditions favoring ("10' final ext") or inhibiting ("no final ext") 3' nontemplated nucleotide addition, *Tne* DNA polymerase produced PCR products that were 95-100% free from nontemplated nucleotide addition ("n") for each locus examined. *Taq* DNA polymerase, however, demonstrated significant addition of nontemplated nucleotides under inhibiting conditions in most loci tested, while under permissive conditions well over half, and in some cases all, of the PCR product produced by *Taq* DNA polymerase demonstrated an additional nontemplated 3' nucleotide. Furthermore, under most conditions the amount of PCR product yielded by *Tne* DNA polymerase was at least as high as that of *Taq* DNA polymerase, and for some loci was 3- to 4-fold higher.

Figure 9 shows two examples of electropherogram gel scans, aligned by PCR product size, comparing the PCR products obtained with *Taq* and *Tne* polymerases with a 10-minute final extension. For the D15S153 locus, *Taq* exhibited non-templated nucleotide addition to 40% of the PCR product (Figure 39), while *Tne* exhibited no such addition of non-templated nucleotides (Figure 9B). Similar results were obtained with the D15S127 locus: 53% of the *Taq* PCR products demonstrated non-templated nucleotide addition (Figure 9C), while none of the *Tne* PCR products demonstrated non-templated nucleotide addition (Figure 9D). These results demonstrate the difficulty in identifying alleles in a heterogeneous pattern as generated by *Taq* amplification, compared to the more homogeneous, simple pattern generated by amplification with *Tne*.

Together with Examples 24-26, these results indicate that *Tne* DNA polymerase and the mutants thereof tested in the present studies are substantially reduced in the ability to add a nontemplated 3' terminal nucleotide to DNA templates, particularly DNA templates comprising microsatellite DNA sequences or STRs. Conversely, *Taq* DNA polymerase demonstrates significant addition of nontemplated 3' nucleotides to PCR products.

TO/290-305860

5

10

15

20

25

Example 28: Comparison of *Taq* and *Tne*

To examine the ability of a truncated form of *Tne* DNA polymerase (N Δ 283, 5'exo-, 10% 3'exo activity) to add a nucleotide to the end of the PCR product, the enzyme was compared side-by-side with wild type *Taq* DNA polymerase in amplifications of short tandem repeats at 5 different marker loci. A portion of ABI Prism Linkage Mapping Set Panel 21 was used for the primer sets for the loci. 15 μ l reactions (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, 200 μ M each dNTP, 333 nM each primer, 60 ng human DNA, 0.1% nonionic detergent, 0.6 U DNA polymerase) were assembled on ice.

Reactions were loaded into a Perkin Elmer model 9600 thermocycler preheated to 95°C and PCR was done using recommended cycling conditions (5 min. pre-denaturation at 95°C; 10 cycles of 15 sec at 95°C, 15 sec at 55 °C, and 60 sec at 72 °C; 20 cycles of 15 sec at 89 °C, 15 sec at 55 °C, and 60 sec at 72°C; 10min final extension at 72°C). A portion of each reaction was diluted, mixed with loading cocktail, heat denatured and loaded on a 8% sequencing gel. The ABI 373 Stretch Automated Sequencer was run for 5-6hr at 15W in order to obtain 1base resolution. Data was analyzed using GeneScan software. Areas of the peaks recognized by the software were used to estimate the percent of extranucleotide addition. Table 7 summarizes the results obtained. Examples of the electropherogram data is shown in Figure 10.

Table 7: Percent extranucleotide addition exhibited by *Taq* and *Tne* DNA polymerases at specific loci.

Locus	<i>Taq</i>(% n+1)	<i>Tne</i>(% n+1)
D16S405	46	0
D16S401	100	45
D16S520	63	0
D15S131	51	0
D16S411	53	0

Example 29: Comparison of The Mutants

In order to evaluate the effect of amino acid substitutions in *Tne* DNA polymerase in regard to extra nucleotide addition, different mutations at position F730 in the untruncated polymerase were compared in side-by-side amplifications with *Tag*(wild type) and a truncated *Tne*(N Δ 219, D323A, F730Y) utilizing a portion of ABI Prism Linkage Mapping Set Panel 21. Six loci were examined. 15 μ l reactions (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, 200 μ M each dNTP, 333 nM each primer, 50-60 ng human DNA, 0.1% nonionic detergent, 0.15-0.6 U DNA polymerase) were assembled on ice.

Reactions were loaded into a Perkin Elmer model 9600 thermocycler preheated to 95 °C and PCR was done using recommended cycling conditions (5 min. pre-denaturation at 95°C; 10 cycles of 15 sec at 95°C, 15 sec at 55 °C, and 60 sec at 72 °C; 20 cycles of 15 sec at 89 °C, 15 sec at 55 °C, and 60 sec at 72°C; 10min final extension at 72°C). A portion of each reaction was diluted, mixed with loading cocktail, heat denatured and loaded on a 8% sequencing gel. The ABI 373 Stretch Automated Sequencer was run for 5-6hr at 15W in order to obtain 1base resolution. Data was analyzed using GeneScan software. Areas of the peaks recognized by the software were used to estimate the percent of extranucleotide addition. Table 8 summarizes the results obtained. An example of the electropherogram data is shown in Figure 11.

Table 8: Percent extranucleotide addition exhibited by mutant *The* DNA polymerases at specific loci.

mutant:	locus:	D16S405	D16S401	D15S131	D15S127	D16S511	D15S153
5	<i>Tag</i> (wild type)	46%	100%	51%	100%	100%	100%
	The-1 (N Δ 219, D323A, F730Y)	0%	0%	0%	0%	0%	0%
	The-35 (D137A, D323A)	0%	52%	0%	0%	0%	0%
	The-18 (D137A, D323A, F730Y)	0%	2%	0%	0%	0%	0%
	The-13 (D137A, D323A, R722H, F730Y)	0%	0%	0%	0%	0%	0%
10	The-14 (D137A, D323A, F730A)	nd	0%	0%	nd	nd	nd
	The-47 (D137A, D323A, F730S)	0%	0%	0%	0%	0%	0%
	The-48 (D137A, D323A, F730T)	0%	0%	0%	0%	0%	0%

Example 30: Comparison of *Tne* and *Taq* Mutants

In order to evaluate the effect of amino acid substitution at position F667 in *Taq* DNA polymerase(equivalent to F730 in *Tne* DNA polymerase) in regard to extra nucleotide addition, a commercially available mutant of *Taq* DNA polymerase (*Taq* FS) (N Δ 3, G46D, F667Y) was compared in side-by-side amplifications with *Taq* DNA polymerase(wild type) and *Tne*-1 DNA polymerase(N Δ 219, D323A, F730Y). Three loci were examined (a portion of ABI Prism Linkage Mapping Set Panel 21). 15 ul reactions (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, 200 uM each dNTP, 333 nM each primer, 60 ng human DNA, 0.1% nonionic detergent, 0.6 U DNA polymerase) were assembled on ice.

Reactions were loaded into a Perkin Elmer model 9600 thermocycler preheated to 95 °C and PCR was done using recommended cycling conditions (5 min. pre-denaturation at 95°C; 10 cycles of 15 sec at 95°C, 15 sec at 55 °C, and 60 sec at 72 °C; 20 cycles of 15 sec at 89°C, 15 sec at 55 °C, and 60 sec at 72 °C; 10min final extension at 72 °C). A portion of each reaction was diluted, mixed with loading cocktail, heat denatured and loaded on a 8% sequencing gel. The ABI 373 Stretch Automated Sequencer was run for 5-6hr at 15W in order to obtain 1base resolution. Data was analyzed using GeneScan software. Areas of the peaks recognized by the software were used to estimate the percent of extranucleotide addition. Table 9 summarizes the results obtained. Examples of the electropherogram data are shown in Figure 12.

102290-38875960

Table 9: Percent extranucleotide addition exhibited by Taq and Tne DNA polymerases at specific loci.

locus	Taq(% n+1)	TaqFS(% n+1)	Tne-1(% n+1)
D16S411	48	0	0
D15S127	100	31	5
D15S153	100	29	0

Example 31: Comparison of Tne Mutants

In order to evaluate the effect of amino acid substitutions at position R722 in *Tne* DNA polymerase in regard to extranucleotide addition, different mutations in the polymerase were compared in side-by-side amplifications utilizing a portion of ABI Prism Linkage Mapping Set Panel 21. Six loci were examined. 15 μ l reactions (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μ M each dNTP, 333 nM each primer, 50-60 ng human DNA, 0.1% nonionic detergent, 0.2-0.6 U DNA polymerase) were assembled on ice.

Reactions were loaded into a Perkin Elmer model 9600 thermocycler preheated to 95°C and PCR was done using recommended cycling conditions (5 min. pre-denaturation at 95°C; 10 cycles of 15 sec at 95°C, 15 sec at 55°C, and 60 sec at 72°C; 20 cycles of 15 sec at 89°C, 15 sec at 55°C, and 60 sec at 72°C; 10min final extension at 72°C). A portion of each reaction was diluted, mixed with loading cocktail, heat denatured and loaded on a 8% sequencing gel. The ABI 373 Stretch Automated Sequencer was run for 5-6hr at 15W in order to obtain 1base resolution. Data was analyzed using GeneScan software. Heights of the n and n+1 peaks recognized by the software were used to estimate the percent of extranucleotide addition. Table 10 summarizes the results obtained. An example of the electropherogram data is shown in Figure 13.

Table 10: Percent extranucleotide addition exhibited by mutant Tn10 DNA polymerases at specific loci.

mutant:	locus:	D16S405	D16S401	D15S131	D15S127	D16S511	D15S153
Tne-35 (D137A, D323A)	0%	54%	0%	0%	0%	0%	0%
Tne-109 (D137A, D323A, R722Y)	0%	0%	0%	0%	0%	0%	0%
Tne-110 (D137A, D323A, R722L)	0%	0%	0%	0%	0%	0%	0%
Tne-114 (D137A, D323A, R722K)	0%	0%	0%	0%	0%	0%	0%
Tne-115 (D137A, D323A, R722Q)	0%	0%	0%	0%	0%	0%	0%
Tne-116 (D137A, D323A, R722H)	0%	0%	0%	0%	0%	0%	0%

Example 32: Generation of *Tne* DNA Polymerase Mutant K726R

The mutation of the *Tne* polymerase was done by essentially the same procedure as described above in Example 13. The single-stranded DNA was isolated from pSport-*Tne* containing D137A and D323A mutations. The 5 oligonucleotide used for the mutagenesis was 5'-GAA GTT CAC CAT ***CCG GCC*** GAC CCG TCG CAT TTC 3' (SEQ ID NO:93). An *Xma*III site (bold italics in the above sequence) was introduced into the oligonucleotide for easy screening of the mutants. The mutation was confirmed by DNA sequencing. The clone was named pTne129 (D137A, D323A, K726R).

10 **Example 33: Determination of the Activity of Non-templated One Base Addition for *Tne* DNA Polymerase and its Mutant D137A, D323A, K726R, by Primer Extension Assay**

15 The mutant *Tne* DNA polymerase (*Tne* D137A, D323A, K726R) prepared in Example 32 was purified as described in Example 20. The assay for non-templated one base addition was conducted as described in Example 23. The results were as follows:

<i>Tne</i> DNA Polymerase	% of Product With N+1
D137A, D323A	78.4
D137A, D323A, R722H	1.7
D137A, D323A, K726R	0.9

20 These results demonstrate that mutation of the lysine residue at position 726 of *Tne*, particularly to arginine, substantially reduces the activity of the polymerase in adding non-templated bases.

Having now fully described the present invention in some detail by way of 25 illustration and example for purposes of clarity of understanding, it will be obvious

to one of ordinary skill in the art that the same can be performed by modifying or changing the invention within a wide and equivalent range of conditions, formulations and other parameters without affecting the scope of the invention or any specific embodiment thereof, and that such modifications or changes are intended to be encompassed within the scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains, and are herein incorporated by reference to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference.

5

10

038932 0027495